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**DEFINING THE AETIOLOGY AND ANTIMICROBIAL
SUSCEPTIBILITY PATTERNS OF THE PREDOMINANT
BACTERIA ASSOCIATED WITH BLOODSTREAM
INFECTIONS AT THE HOSPITAL FOR TROPICAL
DISEASES IN HO CHI MINH CITY, VIETNAM**

by

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A thesis submitted to the Open University U.K

For the degree of Doctor of Philosophy in the field of Life Sciences

Oxford University Clinical Research Unit

Hospital for Tropical Diseases

Ho Chi Minh City, Viet Nam

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Abstract

Bloodstream infections (BSI) are among the most common critical diseases that require intensive care and continuous surveillance. According to many multinational antimicrobial resistance surveillance schemes, Asia represents a substantial reservoir of clinically relevant antimicrobial resistant genes. These genes include extended spectrum Beta lactamases (ESBLs) and AmpC lactamases in the Enterobacteriaceae, methicillin-resistance genes in *Staphylococcus aureus*, carbapenemase-resistance genes in Gram-negative bacilli and vancomycin-resistance genes in enterococcus. Asian countries are also burdened with high prevalence of HIV, in which BSI can be a major health problem in these immunocompromised people. Currently, there are no national data regarding the pathogens associated with BSI in Vietnam. Additionally, there are limited BSI data in specific populations, such as those infected with HIV, and there is limited information regarding the distribution and epidemiology of hospital-acquired and community-acquired infections. Data contributing to our understanding of common antimicrobial resistance mechanisms or profiles, including ESBLs, AmpC, KPC, and MRSA in BSI pathogens in Vietnam are also scarce.

The aims of this study were to describe the characteristics of BSI in the Hospital for Tropical Diseases (HTD), a tertiary healthcare facility for infectious diseases in the south of Vietnam. I aimed to identify the most common BSI pathogens and their antimicrobial resistance profile in the context of the aetiological agent and disease outcome. Further, I aimed to define the specific types of ESBLs and AmpC genes circulating in Gram-negative bacilli isolated from BSI patients. I further describe the clinical and laboratory characteristics of BSI infections caused by the non-typhoidal *Salmonella* (iNTS) a particular group of BSI pathogens that are prevalent in immunocompromised patients. Lastly, I aimed to validate an automatic antimicrobial

susceptibility testing system for *Salmonella* isolates in comparison to manual testing methods. I found that BSI in HTD was characterised by a low annual blood culture positivity rate (7%) and a declining annual trend of mortality. A high proportion of BSI was from patients in the intensive care unit (33%) and the HIV ward (22%). I report an increased trend of multidrug-resistant Gram-negative and Gram-positive pathogens in both hospital-acquired and community-acquired BSI infections.

I additionally report a case study for BSI due to *Vibrio cholerae* non-O1, non-O139 and the first-ever outbreak report of *Brucella melitensis* in Vietnam. I performed molecular characterisation for all Gram-negative organisms isolated over a four-year period that exhibited reduced susceptibility against 3rd cephalosporin. Phenotypic screening found 304/1,017 (30%) organisms that were resistance to third generation cephalosporins; 172/1017 (16.9%) of isolates exhibited ESBL activity, 6.2% (63/1017) had AmpC activity, and 0.5% (5/1017) had both ESBL and AmpC activity. *E. coli* and *Aeromonas* spp. were the most common organisms associated with ESBL and AmpC phenotypes, respectively. There was no significant difference ($p>0.05$) between antimicrobial resistance phenotypes of organisms associated with community and hospital-acquired infections. I retrospectively identified 102 cases of iNTS infections in HTD between 2008 and 2013. Of 102 iNTS patients, 71% were HIV-infected, >90% were adults, 71% were male, and 33% reported intravenous drug use. Twenty-six/92 (28%) patients with a known outcome died; HIV infection was significantly associated with death ($p=0.039$). *S. Enteritidis* (Sequence Type (ST)11) (48%, 43/89) and *S. Typhimurium* (ST19, 34 and 1544) (26%, 23/89) were the most commonly identified serovars; *S. Typhimurium* was significantly more common in HIV-infected individuals ($p=0.003$). Through comparison of different antimicrobial testing methods for *Salmonella*, I identified a problematic agreement or errors were with quinolone antimicrobials. I found that the VITEK automatic antimicrobial susceptibility testing system did not

produce satisfactory results for the *Salmonella*. However, the disk-diffusion method was a more reliable method for testing beta-lactams, azithromycin, and trimethoprim-sulfamethoxazole. In conclusion, BSI in HTD are characterised with an increasing trend of multi-drug resistant organisms that will challenge clinical and laboratory diagnostic as and future treatment options.

Co-Authorship

The work contained in this thesis was performed primarily by me, under the supervision of my supervisors, during the four-year and a half year of my PhD project. Colleagues from Hospital for Tropical Diseases (HTD), Microbiology Laboratory and Enteric Group also contributed to this work as described below.

In the retrospective study of bloodstream infection in HTD during 2010-2014 (Chapter 3), I have designed the study protocol and collected all the clinical and laboratory data by myself. Data analysis was mainly performed by myself with the help of Mr. Raphael Zellweger and Mr. Trinh Van Tan in Enteric Group. In the study of outbreak of *Brucella melitensis*, I contributed on laboratory identification and antimicrobial susceptibility testing. I have developed, designed and analysis the study of ESBL and AmpC detection genes in Gram negative bacilli (Chapter 4). The molecular tests were done mostly by Mr. Nguyen Huu Hien (HTD). In Chapter 5, the retrospective study of nontyphoidal *Salmonella* infection was mainly developed and performed by me. Data entry was input by The Clinical Trial Unit (OUCRU-VN). I have learnt and worked on MLST techniques with Ms Le Thi Phuong Tu (Enteric group). Most the work on Chapter 6 about different antimicrobial testing methods was completed by me, except for testing for *Salmonella* Paratyphi A strains was performed by Ms Le Thi Quynh Ngan (HTD) as a part of her Master degree. Throughout the study period, I was closely supervised and assisted by my principle supervisor, Professor Stephen Baker.

Publications

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Lan, Nguyen Phu Huong, Tran Vu Thieu Nga, Nguyen Thi Thu Yen, Le Thi Dung, Ha Thanh Tuyen, James I Campbell, Jamie Whitehorn, Guy Thwaites, Nguyen Van Vinh Chau, and Stephen Baker. 2014. “Two Cases of Bacteriemia Caused by Nontoxigenic, Non-O1, Non-O139 *Vibrio Cholerae* Isolates in Ho Chi Minh City, Vietnam.” *Journal of Clinical Microbiology* 52 (10). American Society for Microbiology: 3819–21. doi:10.1128/JCM.01915-14.

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Declaration

Other than the assistance outlined in the acknowledgements, the work described in this thesis is my own work and has not been submitted for a degree or other qualification to this or any other university.

Abbreviations

AMK	Amikacin
AMR	Antimicrobial resistance
ANSORP	Asian Network for Surveillance of Resistant Pathogen
ART	Antiretroviral therapy
AST	Antimicrobial susceptibility testing
AUG	Amoxicillin-clavulanate
BHI	Brain heart infusion
BMD	Broth micro dilution method
BSAC	British Society for Antimicrobial Chemotherapy
BSI	Bloodstream infection
C	Chloramphenicol
CA	Category agreement
CA-BSI	community-acquired bloodstream infection
CAI	community-acquired infection
CIP	Ciprofloxacin
CDC	Center for Disease Control and Prevention
CLSI	The Clinical Laboratory Standard Institute
CNS	Coagulase-negative <i>Staphylococcus</i>
CRE	Carbapenem-resistant <i>Enterobacteriaceae</i>
CT	Cholera toxin
DNA	Deoxyribonucleic acid
ECDC	The European Centre for Disease Prevention and Control
ESBL	Extended-spectrum- β lactamases
ETP	Ertapenem

EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FEP	Cefepime
FOX	Cefoxitin
HAART	Highly active antiretroviral therapy
HA-BSI	Hospital-acquired bloodstream infection
HAI	Hospital-acquired infection
HCMC	Hochiminh city
HIV	Human immunodeficiency virus
HTD	Hospital for Tropical Diseases
I	Intermediate
IMP	Imipenem
iNTS	Invasive nontyphoidal <i>Salmonella</i> infection
IQR	Interquartile range
ICU	Intensive care unit
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
LCBI	laboratory confirmed bloodstream infection
MALDI-TOF	Matrix assisted laser absorption ionization-time of flight mass spectrometry
MD	Major discrepancy
MDR	Multidrug-resistant
MEM	Meropenem
MIC	Minimum inhibition concentration
MJD	Major discrepancy
MnD	Minor discrepancy
MLST	Multilocus Sequence Typing

MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MLVA	Multiple-locus variable number tandem repeat analysis
NA	Nalidixic acid
NHSN	National Healthcare Safety Network
NTS	Non-typhoidal <i>Salmonella</i>
OFX	Ofloxacin
OUCRU	Oxford Clinical Research Unit
PCR	Polymerase chain reaction
PDR	Pan-drug resistant
PCT	Procalcitonin
PFGE	Pulsed-field gel electrophoresis
qSOFA	quick Sepsis related organ failure assessment
R	Resistant
RNA	Ribonucleic acid
S	Susceptible
SIRS	Systemic inflammatory response syndrome
SMART	The Study for Monitoring Antimicrobial Resistance Trends
ST	Sequence type
SXT	Trimethoprim - sulfamethoxazol
TZP	Piperacillin-tazobactam
USA or US	The United State of America
VN	Vietnam
VMD	Very major discrepancy
VRE	Vancomycin-resistant <i>enterococcus</i>
WHO	World Health Organization
XDR	Extensively-drug-resistant

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Chapter 1

Literature Review

1.1 Bloodstream infections (BSI)

Typically, blood is a sterile body environment, which is free from microorganisms. A Bloodstream infection (BSI) is defined by the presence of microorganism (bacteria or fungi) circulating in the blood. BSI can be classified by duration of bacteria presenting in the blood as transient bacteraemia (few minutes or hours), intermittent bacteraemia (intermittent isolation of the same bacteria from one patient) and persistent bacteraemia. The diagnosis of BSI is usually established by a positive blood culture taken from infection-suspected patients when contamination has been ruled out (Laupland et al., 2013; Viscoli, 2016).

1.2 Laboratory-confirmed BSI (LCBI)

For the purposes of surveillances CDC/NHSN developed criteria to identify laboratory-confirmed BSI (LCBI) for all types of patients(NHSN, 2018). LCBI must meet at least one of the three criteria: a) a recognized pathogen cultured from blood which is not related to an infection at another site, b) the presence of one or more of the following signs or symptoms: fever ($>38^{\circ}\text{C}$), chill or hypotension with a positive laboratory results and not related to an infection at another site, c) common commensal bacteria cultured from two or more blood cultures drawn on separate occasions. It is noted that various elements must occur within one day while considering the LCBI diagnosis.

1.3 Classification of BSI

For the purposes of clinical management and antimicrobial treatment, BSI are classified based on place of acquisition (Australian Infection Control Association, 2003; Laupland and Church, 2014) and on the characteristics of the host (Laupland and Church, 2014; Viscoli, 2016).

1.3.1 Classification by place of acquisition

If classification is made on the place of acquisition, BSI can be categorized into groups as community-acquired BSI, Hospital-acquired BSI, or maternally-acquired BSI (Anderson et al., 2014; Hugonnet et al., 2004). Community-acquired BSI is defined as BSI occurring in an outpatient or first identified (culture drawn) <48 hours following admission to hospital. Hospital-acquired BSI is the BSI that is first identified (culture drawn) ≥48h after hospital admission and within 48 hours following hospital discharge. Lastly, maternally-acquired BSI is BSI that appears <48 hours after birth in an infant. (Australian Infection Control Association, 2003)

1.3.2 Classification by characteristics of the host

If classification is made on the characteristics of the host, BSI is classified as in immunocompetent patients with intact defences, in new-borns, or elderly patients with impairing defences, and in immunocompromised patients with deficient defences (HIV-infected patients, patients on immunosuppression, cytotoxic therapy etc.) (Viscoli, 2016). To establish a BSI case, recent definitions combine microbiology results (mostly blood culture) together with the clinical signs of infection. However, it is not always easy to exclude contamination and the clinical signs of infection are not specific.

1.4 The manifestations of BSI

BSI has a variety of clinical manifestations, which can be presented in a single symptom like fever or a systematic severe disease like septic shock. Although some asymptomatic BSI cases have been documented, most BSI cases have specific signs and symptoms. In this case, BSI is called sepsis.

1.4.1 Sepsis

According to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), sepsis is defined as “life-threatening organ dysfunction caused by a dysregulated host response to infection” (Singer et al., 2016). In general hospital, sepsis can be quickly approached via a new bedside clinical quick score (qSOFA): respiratory rate more than 21 times per minute, altered mentation or systolic blood pressure less than or equals to 100 mmHg. Notably, qSOFA is a tool to assess sepsis, not septic shock in Sepsis-3.

In Sepsis-3, the systemic inflammatory response syndrome (SIRS) was no longer used to diagnosed sepsis because its criteria were unspecified for sepsis and can result from other many conditions such as trauma, ischemia...However, SIRS was advised to use for general diagnosis of infection. Clinical SIRS is established if two or more criteria are met: body temperature $>38^{\circ}\text{C}$ (100.4°F) or $<36^{\circ}\text{C}$ (96.8°F); heart rate >90 beats per minute; hyperventilation (respiratory rate >20 breaths per minute or arterial carbon dioxide tension (PaCO_2) of <32 mm Hg); white blood cell count ($>12,000/\mu\text{L}$, or $<4,000/\mu\text{L}$, or $>10\%$ immature band forms)(Singer et al., 2016).

1.4.2 Septic shock

Septic shock is a severe and late stage of sepsis. The most recent definition was also established in The Sepsis-3 consensus as “Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality”(Singer et al., 2016). Patients with septic shock can be identified with a clinical construct of sepsis with persisting hypotension requiring vasopressors to maintain a mean arterial pressure ≥ 65 mmHg and having a serum lactate level >2 mmol/L (18mg/dL) despite adequate volume resuscitation.

1.5 The epidemiology of BSI

BSI is well-known severe disease with high morbidity and mortality worldwide. It remained the 11th leading causes of death in 2013 - 2014 in the US (Kochanek et al., 2016; Xu et al., 2016) and in the top seven causes of death in the US and Europe combined (Goto and Al-Hasan, 2013). Identifying the importance of bloodstream infection, population-based studies or longitudinal respective surveillances have been made in many countries, but these originate mostly in Europe and North America. Although there are differences in methods, populations and time, data in these various studies showed that BSI was a heavy burden with high incidence, mortality, and fatality. The annual incidence of BSI ranged from 40 to 257/100,000 persons-year in population-based studies in which incidence in most was $>150/100,000$ persons-year (Goto and Al-Hasan, 2013; Laupland et al., 2013; Mehl et al., 2017; Uslan, 2007; Wisplinghoff et al., 2004).

1.5.1 Geographical distribution

1.5.1.1 America

In the US, despite the mortality data for notifiable diseases being reported annually, the most recent population-based study was conveyed in Olmsted County, Minnesota since 2003 (Uslan, 2007). The annual incidence was 189/100,000 persons-year with mortality rate of 13.5%. Comparatively, in Canada, Laupland *et al.* conducted a population-based study for bloodstream infections for five years (2000-2005) in the Calgary Health Region; this study included community-onset BSI only. The overall annual incidence was 81.6/100,000 persons-year, which was lower than in the US. However, the fatality rate was the same as in the US at 13% (Gregson and Church, 2007). Further analysis on severe BSI cases was conducted in the same population in 2010-2012. The annual incidence of severe BSI was 15.7/100,000 people while the case-fatality was report to be 6.5/100,000 populations (Laupland et al., 2004).

1.5.1.2 Europe

Several countries in Europe have conducted population-based BSI studies; most were conducted before 2010. In Denmark, BSI was the seventh most common cause of death (Goto and Al-Hasan, 2013). A study conducted over 15-years (1992-2006) in North Denmark showed a remarkable increase in BSI incidence from 120 to 201/100,000 person-year over this period. This increasing trend was observed in both community and hospital acquired BSI groups. In contrast, the mortality rate reduced with time, beginning at 22.7% during 1992-1996 to 20.6% during 2002-2006, this was mostly attributed to the reduction of mortality rate in community-origin BSI(Søgaard et al., 2011). A further population based study from Funen, Denmark in 2000-2008 found a higher incidence rate of 215.7/100,000 persons-year, but the trend declined by 3.7% annually (Nielsen et al., 2014).

Finland conducted a BSI study at a country level during 2004-2007 that investigated >30,000 BSI episodes. The annual BSI incidence was 159/100,000 person-year with a

significant average increase of 4.4%/year. The mortality rate was approximately 21/100,000 persons-year which increased 4% every year (Skogberg et al., 2012).

Comparable to Denmark, BSI was the seventh leading causes of death in Finland (Goto and Al-Hasan, 2013).

A further population-based conducted over 12 years (2002-2013) in Mid-Norway investigated 1,995 episodes of BSI in adult patients. The incidence rate was as high as 215/100,000 person-years, which was higher in male and elderly group (Mehl et al., 2017). The overall mortality rate 32/100,000 person-years, decreasing up to 29/100,000 person-years in the last period of the study (2008-2013).

The most recent multi-centre population-based study in Europe took place in Switzerland from 2008 to 2014 as part of the National Surveillance for BSI. Although there was no information about the annual incidence, the number of BSI episodes increased from 5,754 to 6,694 in 2008 and 2014, respectively. The mortality rate was not documented in this study (Buetti et al., 2016). In order to have a broader view of BSI in Europe, a recent point-prevalence survey in 29 European countries showed that BSI was the most common healthcare-associated infection (45%) in 2011-2012 (Zingg et al., 2017).

1.5.1.3 Australia

There are no population incidence for BSI in Australia (Bloodstream et al., 2012; Laupland et al., 2013), there are few studies about the rate and characteristics of BSI. In a 350 bed-tertiary hospital in Melbourne, the number of patients with microbiologically-confirmed BSI was >2,000 over a 9 years period (Akova, 2017). A similar study in Townsville hospital over a 10 year had a bacteraemia rate of 10.12 per 1,000 admissions (Porter et al., 2013).

1.5.1.4 Sub-Saharan Africa

Like Australia, there are limited data the incidence of BSI in Africa. However, many African countries have conducted retrospective studies about BSI which highlighted the importance of BSI across the continent (Moyo et al., 2010; Obeng-Nkrumah et al., 2016; Reddy et al., 2010; Tariq, 2014). According to a systematic review of >20 studies about BSI during 1984-2006 in Africa, there were >58,000 patients receiving a blood culture request for BSI diagnosis. Among them, laboratory-confirmed BSI cases were as many as 5,578 cases (9.6%). A specific characteristic of BSI in Africa was that many patients were co-infected with malaria, HIV, or *M. tuberculosis*. In 16 studies with fatality rate data, the overall death rate was 18.1 % due to BSI, which is considerably higher than in patients without BSI (Reddy et al., 2010). Hospital based research from some African hospitals demonstrated a high prevalence of positive blood cultures. In a referral hospital in Ghana, the overall positive blood culture rate was 9.3% with a higher prevalence in the elderly (13.3%) and infants (20.9%)(Obeng-Nkrumah et al., 2016).

1.5.1.5 Asia

Unlike Europe and North America, there are little data about BSI at a population level. However, many countries have published data regarding notifiable issues related to BSI.

Thailand

Thailand is one of the few countries in Asia that has population-based data for BSI bloodstream infection. According to multicentre surveillance in northeast Thailand over seven years, the overall incidence rate for all types of BSI was 31/100,000 person-years, which increased over time. CA-BSI were as many as 73% of total cases while HA-BSI

were only 16%. In the CA-BSI group, highest incidence was found in infants and the elderly (83 and 221/100,000 person-years, respectively). Males were more likely to have CA-BSI than females (34 vs. 28/100,000 person-years, $p < 0.001$) (Kanoksil et al., 2013). The prevalence of nosocomial BSI in Thailand was approximately 6.5% in 2006. A report from a hospital in the country measured the prevalence of hospital bacteraemia as 11.6/1000 admissions in 2012. 22.5% cases developed septic shock, while the mortality rate was 28.3% (Chusri et al., 2012).

Cambodia

In Cambodia, BSI surveillance has been conducted for children and as adults. In the pediatric study, the incidence rate rose from 14/1,000 to 50/1,000 admission during 2007-2011. Community acquired BSI was predominant (89%). The mortality rate was as high as 19%, particularly in neonates (Stoesser et al., 2013). Surveillance in adults over the same period (2007-2010) was focused on 463 laboratory-confirmed BSI cases. The rate of CA-BSI in adult was comparable to children (89% and 86%, respectively). Common underlying diseases in adults were Human Immunodeficiency Virus infection (HIV) and diabetes. One notable piece of data from this study was antimicrobial consumption prior hospitalization, which was reported in 22% cases (Vlieghe et al., 2013).

1.5.2 Sex

In most BSI studies, males have a higher incidence of BSI than female (Uslan, 2007). In a population based study from Olmsted County, Minnesota, USA reported that the BSI incidence in elderly patients was twice as high in males than women (Uslan, 2007).

1.5.3 Length of ICU stay and hospitalization for BSI

One of the major impacts of bacteraemia is the extra length of stay in the healthcare facility. In 1990s, Crowe and his researchers compared the length of Intensive care unit (ICU) stay from different illnesses. BSI patients had four-times longer stay in the ICU than the other illnesses (12 days vs. 3 days) (Crowe et al., 1998). In a case-control study from Scotland, BSI patients had higher ICU stay than a control group (17 days vs. 7.8 days, $p=0.001$). Data in this study originated from an adult hospital during 2011-2013 (Brooks et al., 2016) Another study in nine public hospitals in Queensland, Australia in 2013 also showed a significantly longer ICU stays in patients with bacteraemia than those without (15.5 days vs. 4 days, $p<0.001$)(Barnett et al., 2013). An additional length of hospital stay was also found in BSI patients who were discharged alive.

1.5.4 Seasonal variation in BSI

Seasons tend to affect the epidemiology of BSI. In Africa, a shift of the predominant pathogens associated with BSI has been observed in the sunny and rainy season. Many African countries such as Malawi, Egypt, Gambia have non-typhoidal *Salmonella* as a main cause of BSI in the wet season, while *S. pneumoniae* is the commonest cause of BSI in the dry season(Bell et al., 2001; Reddy et al., 2010).

The incidence of *E. coli* BSI also exhibits seasonal variation. In a population-based study from the US, the incidence rate of in BSI increased by up to 35% in the warm months (June -September). This study concluded that for every 5°C increased in temperature, incidence rate of BSI increased by 7%(Al-Hasan et al., 2009). In Israel, an eight-year study investigated 983 BSI cases caused by between 2001 and 2008; the rate of in BSI was significantly higher in summer than in the other seasons (31+/-6 days vs. 28+/-6, 27+/-4, $p<0.001$, respectively)(Chazan et al., 2010). In tropical regions, the prevalence of some infectious diseases increases in rainy season. One supportive

example is typhoid fever, a well-known bacteraemia caused by *Salmonella Typhi*.

Typhoid fever is more common during periods of rainfall. A 4-year study conducted in Kathmandu, Nepal showed that the typhoid cases correlated with rainfall. July was the peak of *S. Typhi* as well as the peak of the wet season (Karkey et al., 2010). In Europe and US, there were found to be more severe sepsis cases (35% higher in the Europe and 17% higher in the US) in cold months (fall and winter season) of the year (Mayr et al., 2014). This may be due to increasing respiratory infection incidence in cold weather, which can lead to sepsis in some risk group patients. However, the season had no impact on the nosocomial infections. In a nationwide surveillance of nosocomial infection in the US, there were no changes of causative agent in BSI infection due to seasonal variation (Wisplinghoff et al., 2004).

1.5.5 Source of infection

Pneumoniae is the most common source of BSI globally, both in community-acquired and hospital -acquired infection. In a CA-BSI study, pneumoniae was found in 15% of all cases which was characterized by common respiratory pathogens (*Streptococcus pneumoniae*, *Staphylococcus aureus*)(Timsit et al., 2014). Urinary tract infections are another common source of BSI. In a population-based study from Norway, the rate of BSI from the urinary source was 81/100,000 person-years, which was higher than from the respiratory tract and biliary tract (Mehl et al., 2017). In Australia, urinary infection was the most common primary site of BSI with the percentage of 33% in all cases over ten years(Lim et al., 2014). However, there were a considerable proportion of primary BSI cases with an unidentified source of infection. Primary BSI was as many as 53% of all nosocomial cases reported in a nationwide US surveillance study and 29% of all community -acquired BSI cases in a study from Australia (Wisplinghoff et al., 2004; Lim et al., 2014;). Infection via catheter is also a common infection site for nosocomial BSI(Tan et al., 2007; Timsit et al., 2012). Other primary sources of BSI infection have

been reported as being from an abdominal origin, wound and soft tissue abscesses (Lim et al., 2014; Son et al., 2010).

1.5.6 Community- acquired infections and hospital-acquired infection

Based on the place of acquisition, BSI can be divided into two main categories: community-acquired infection(CAI) and hospital-acquired infection (HAI). These definitions are widely used for the clinical management of BSI. There are certain groups of patients who are at risk of CAI or HAI and there are various bacteria causing CAI or HAI. Hence, clinical care and antimicrobial regime for each group are also different.

The time-point 48 hours after hospitalization is used to distinguish between community and nosocomial infection. Community-acquired infection, or community-associated infection can be defined as infection origins in the community or happens in the hospital within 48 hours after admission. According to this definition, community-acquired BSI is diagnosed if at least one positive blood culture is collected during 48 hours after admission and excluding the contaminants. Hospital-acquired BSI (HA-BSI) can be determined if at least one blood culture grew with pathogenic microorganism after 48 hours of admission(Goto and Al-Hasan, 2013; Gregson and Church, 2007; Hugonnet et al., 2004; Kang et al., 2012; Nagao, 2013).

1.5.6.1 Community-acquired BSI:

Epidemiology

A large proportion of BSI originates from the community. The National BSI Surveillance programme in Switzerland from 2008-2014 found that 70% of BSI were CA-BSI (Buetti et al., 2016). The prevalence of CA-HAI increased from 39% to 45% over 7 years. In a Norway population- based study, CA-HAI incidence was much higher

than HA-BSI (102 vs. 30/100,000 persons-year)(Mehl et al., 2017). The rate of CA-HAI also increased from 36 to 40/100,000 bed-days in 12 years. Mortality rate in CA-BSI group was 10%, which was higher than in HA-BSI group (6%). In Denmark, 47% of all BSI were CA-BSI according to a population based cohort study(Søgaard et al., 2011). Mortality rate in CA-BSI decreased from 19% to 15.4% from 1992 to 2006. A multicentre study in Thailand regarding CA-BSI was conducted in 2004-2010. CA-BSI accounted for 73% of all BSI cases in the study time. The incidence rate for CA-BSI increased significantly year by year with an average of 31/100,000 persons-year. The mean mortality rate was 37%, which decreased over time (Kanoksil et al., 2013).

Risk factors for CA-BSI

There were certain groups of people who are at greater epidemiological risk of community-acquired bloodstream infection.

Diabetes mellitus increases the risk of CA-BSI in severe ICU patients. An observational cohort study concluded that diabetes was a predictor of CA-BSI. CA-BSI and sepsis were found significantly higher in patients with diabetes (OR 1.42, $p=0.02$; OR1.26, $p=0.02$, respectively) than patients without. High HbA1C level ($>6.5\%$) was also increased the risk of BSI (McKane et al., 2014). A further group of patients who were at risk CA BSI is those infected with HIV. In an epidemic area of HIV, community-acquired BSI was found more frequently in HIV-infected patients than those without HIV. In Africa, a systematic review showed a high prevalence of community acquired BSI due to non-typhoidal *Salmonella* or fungi in HIV-infected people(Reddy et al., 2010). In Southeast Asia, the prevalence of CA-BSI was as high as 1 in 35 HIV-patients enrolled in public healthcare services in Cambodia, Thailand and Vietnam. Notably, this study found that immunosuppression was closely linked to CA-BSI. HIV-infected

patients with CD4 counts <100 had high prevalence of CA-BSI while there was no CA-BSI case in those on ART treatment(Jay K Varma et al., 2010).

1.5.6.2 Hospital-acquired BSI

Epidemiology

Hospital -acquired infection, or nosocomial infection is defined as infection arising 48 hours after admission. Therefore, hospital-acquired BSI is diagnosed if there is at least one blood culture taken after two days from admission grow pathogenic organism.

Although the proportion of nosocomial BSI is often smaller than community acquired BSI, nosocomial BSI is healthcare burden due to its high cost, fatality and mortality(Kaye et al., 2014; Laupland et al., 2002; Pittet et al., 1994) . Many countries have large surveillances to obtain epidemiology, clinical and microbiology data about nosocomial BSI in a purpose to set up proper management for this disease. Nosocomial BSI usually is usually associated with 7%-39% of all BSI (Goto and Al-Hasan, 2013; Johannes and Marlborough, 2008; Uslan, 2007); therefore the rate of nosocomial BSI is typically lower than the rate of community-origin BSI(Buetti et al., 2016; Goto and Al-Hasan, 2013; Kiertiburanakul et al., 2012; Mehl et al., 2017; Uslan, 2007).

There is a rising trend of HA-BSI with time in many countries. In Europe, both population based-studies in Norway and Denmark showed an increase in HA-BSI rate with time. The annual incidence rate of HA-BSI increased from 36 to 40 episodes/100,000 bed-days(Mehl et al., 2017) during 2002-2013 while the incidence was from 45 to 65/100,000 bed-days in Denmark during 1992-2005(Søgaard et al., 2011). In the US, the Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) study reported >24,000 hospital-acquired BSI during 1995-2002 in 42 hospitals across the country. The annual incidence of hospital-acquired BSI was

60/100,000 hospital admissions while the crude mortality rate was 27% (Wisplinghoff et al., 2004).

There are some patients who are at higher risk of hospital-acquired BSI than the others. Some underlying diseases make the host vulnerable to HA-BSI. Common underlying diseases includes malignancies, neurologic disorders, cardiovascular diseases, gastrointestinal respiratory and renal diseases (Al-Otaibi et al., 2016; Marra et al., 2011; Wisplinghoff et al., 2004). According to a multicentre surveillance for nosocomial infection in the US, patients with malignancies accounted for 10.3% of all nosocomial BSI. Among them, those with neutropenia (mostly due to primary neoplastic disease) had a higher crude mortality rate than the others (36% vs. 31%, $p=0.053$) (Wisplinghoff et al., 2003).

Potential risk factors for HA-BSI

There are various potential risk factors for HA-BSI, which includes plastic catheters, ICU admission, and underlying diseases. Plastic device, such as intravenous catheters, are a well-known predisposing factor for HA-BSI. Although plastic devices play an important role in intensive care, they also act as an entry for bacteria to the blood circulation. Therefore, patients who have plastic devices during hospitalization have high risk of nosocomial infection, including HA-BSI. In Brazil, 70% of nosocomial BSI patients had a central venous catheter and 41% of them also had a urinary catheter (Marra et al., 2011). In a longitudinal study in Spain, the incidence of HA-BSI due to central venous catheter increased from 10/100,000 to 31/100,000 patient-days during 1992-2008 (Marcos et al., 2011).

In the US, intravenous catheters have been considered as a great predisposed factor for HA-BSI, therefore any case with central line-related BSI should be reported (Reddy et

al., 2017). In 2004, the SCOPE study listed intravascular catheters (including central, peripheral and arterial catheters) are the most frequent predisposed factors for HA-BSI (Wisplinghoff et al., 2004). A multicentre study showed that nearly 80% of HA-BSI in elderly patients were catheter-related (Kaye et al., 2014). Another study found that catheter-related BSI occurred in >250,000 patients per year and the average mortality attributed to central-venous catheters was 20% (Guggenbichler et al., 2011). However, the Center for Disease Control and Prevention (CDC) reported that there was a recent decrease in central line-associated BSI to 46% in the US (Reddy et al., 2017).

In France and Japan, intravascular catheters were the top cause of HA-BSI (29% and 43%, respectively) as described in their national multicentre surveillance (Nagao, 2013; Timsit et al., 2012).

In Southeast Asia, central line associated-BSI is an important cause of HA-BSI. Review data from 6 studies in Malaysia, Thailand and Philippines showed the pooled incidence density was 4.7/1000 catheter-days (Ling et al., 2015).

Besides intravenous catheters, other studies showed that surgery or invasive interventions (hemodialysis, mechanic ventilator support) predispose patients to HA-BSI (Arnoni et al., 2007; Gauna et al., 2013; Guggenbichler et al., 2011; Pfeiffer et al., 2006; Prowle et al., 2011; Rello et al., 1994). Moreover, patients who are admitted to the intensive care unit (ICU) have a higher risk of HA-BSI. More than half of HA-BSI occurs in the ICUs (Marra et al., 2011; Wisplinghoff et al., 2004) with an incidence of 5-19 /1,000 patient days (Laupland and Church, 2014; Pittet et al., 1994; Rello et al., 1994). The crude mortality in HA-BSI patients varied from 21% to 66% (Hoenigl et al., 2014; Prowle et al., 2011; Rello et al., 1994; Søgaard et al., 2011). Underlying diseases or chronic diseases can be frequently found in ICU patients who developed CA-HAI later (Wisplinghoff et al., 2004). Risk factors for ICU-acquired BSI were identified as

raised APACHE III score, renal replacement therapy, liver disease, and surgery (Prowle et al., 2011). Other hospital wards that have a high rate of HA-CAI are: surgical units, pediatric units, organ-transplant department, internal medicine, and oncology unit (Marra et al., 2011; Nagao, 2013; Wisplinghoff et al., 2004)

1.5.7 Patient groups and outcome of BSI

1.5.7.1 Critically-ill patients

Critically ill patients and BSI have a close relation. There is a high rate of BSI in patients with critical conditions such as traumatic injuries, renal failure, chronic pulmonary diseases, last stage of AIDS, hepatitis, organ-transplantation, and malignancies (Kiertiburanakul et al., 2012; Mayr et al., 2014; Wisplinghoff et al., 2003). These patients need intensive support such as intubation, intravenous catheter, and parental nutrition, which predispose them to a high risk of nosocomial infection. Treatment regimens require broad-spectrum antimicrobial or combination therapy because nosocomial pathogens are generally highly resistant to common antimicrobials. Moreover, bacteraemia in critical ill patients can progress to severe stages such as severe sepsis and septic shock. In these critical stages, the fatality rate can be >50% (Martin, 2012).

1.5.7.2 HIV infected patients

BSI is a major health problem in HIV-infected people. In areas Africa where HIV is epidemic, BSI is commonly associated with HIV infection. According to a systematic review of BSI in Africa, data generated from 17 studies showed that more patients with BSI in HIV groups than in non-HIV infected groups (34.5% vs. 15.2%, $p < 0.001$) (Reddy et al., 2010). This review also reported the prevalence of HIV-infection was 18.5% in pediatric patients and 53.5% in adults who was presented with BSI on admission.

Most BSI in HIV infected patients is community-associated. The mortality rate in HIV infected patients ranges from 7%-46%, which is higher in developing countries(Huson et al., 2014). A common risk factor for BSI were associated with HIV infection is a CD₄ count <200 cells/mm³, antiretroviral therapy is protective (Kiertiburanakul et al., 2012; Moyo et al., 2010; Reddy et al., 2010; Jay K. Varma et al., 2010).

Several African countries have conducted studies regarding BSI in relation to HIV status. All of them report a high morbidity and mortality rate due to BSI in HIV infected participants. A survey from Nigeria, where the HIV rate is approximately 6%, showed that BSI rate was 18.5% in all HIV-seropositive patients(Kolo et al., 2015). In Moshi, northern Tanzania, HIV-seropositive was identified in 39% of all adult patients with BSI. The death rate in those infected with HIV was significantly higher than in those without HIV infection (19.8% vs. 4.8%, $p<0.001$)(John A. Crump et al., 2011). In Nairobi, Kenya during 1988-1997, 38% of HIV infected admissions to a national hospital had bloodstream infection. The mortality rate of BSI in HIV infected patients in this hospital reduced significantly from 58% to 18.5% after a new treatment regime for tuberculosis infections was introduced(Arthur et al., 2001). The most recent sentinel surveillance of bacteraemia was done in Malawi during 1998-2016. This study marked the decreased incidence of bloodstream infection (327/100,000 in 1998 to 120/100,000 in 2016; $p<0.0001$) in concordance with the increased enrolment on the ART program (2.3% in 2004 to 67% in 2014) in this country during the investigation time (Musicha et al., 2017).

Besides Africa, Southeast Asia is also an epidemic area for HIV-infection. Reports about BSI in HIV infected individuals are available from Thailand, Cambodia, and Vietnam. According to an international study in Southeast Asia, bacteraemia was found

in 1/35 out patients who were seropositive for HIV. In this patient group, the median CD₄ count was significantly lower in HIV patients with BSI than in those without (15 vs. 261 cells/mm³, $p < 0.001$) (Jay K. Varma et al., 2010). In Thailand, a retrospective cohort study regarding BSI in HIV patients over 5 years showed that nearly 90% of the BSI cases were community acquired. The predominant sex was male and the crude mortality was 21% (Kiertiburanakul et al., 2012). In the US, although the prevalence of HIV-infected cases is lower than in Africa and Asia, the rate of severe sepsis in HIV people has increased overtime. Notably, most BSI cases in HIV patients in the US are nosocomial or healthcare associated, whereas in Asia and Africa these infections arise in the community (Kiertiburanakul et al., 2012; Mayr et al., 2014).

CD₄ counts have an inverse relationship with the prevalence of BSI in HIV patients. Patients with CD₄ <200 cells/ μ l had a higher rate of BSI than those with CD₄ >200 cells/ μ l (Adeyemi et al., 2010; Kolo et al., 2015; Jay K. Varma et al., 2010). CD₄ count <100 cells/ μ l act as a predicting factor for tuberculosis and fungal infection (Kiertiburanakul et al., 2012). Even on antiretroviral therapy (ART), HIV-seropositive patients can develop BSI if CD₄ is <100 cells/ μ l (Meynard et al., 2003). ART is a protective factor for HIV-infected person for BSI. After the era of highly active antiretroviral therapy (HAART), the prevalence of BSI in HIV-infected patients reduced significantly globally. A prospective case-control study showed that incidence of bacteraemia reduced from 11 to 8/1,000 person-years during this period ($p < 0.001$) (Tumbarello et al., 2000). In another case-control study from France, rate of BSI fell from 10.5 to 5.5/1,000 hospitalizations before and after HAART era (Meynard et al., 2003). In Southeast Asia, a study about BSI in HIV-infected outpatients showed that no patient on ART developed BSI over the period of investigation (Jay K. Varma et al., 2010).

1.6 Pathogens associated with BSI

The epidemiology of pathogens causing BSI has changed with time. The main changes are a shift towards Gram-negative organisms and the revolution of multi-drug resistant organism from both a community and a hospital origin. The causative agents of BSI include a wide variety of bacteria and fungi, of which bacteria predominate. Of the bacteria, the Gram-negative bacilli are usually the main pathogens for BSI. Common Gram-negative bacilli that cause BSI include, *Klebsiella*, *Salmonella*, *Pseudomonas*, and *Acinetobacter*. Gram-positive bacteria are also important BSI pathogens.

Streptococcus pneumoniae and *Staphylococcus aureus* are the two most notable pathogens associated with BSI in this group.

If the primary infection site is from the urinary tract or intra-abdominal source, then Gram-negative bacilli, especially members of the Enterobacteriaceae are the main pathogens (Mehl et al., 2017; Timsit et al., 2014). Gram-positive organisms, such as *Streptococcus pneumoniae* are more prevalent if the source of BSI is from community-acquired pneumoniae (Timsit et al., 2014). Some studies have shown that BSI due to Gram-negative bacilli is more common in females whereas Gram-positive cocci are more common in males (Mehl et al., 2017). According to nosocomial BSI surveillance in the USA, Gram-positive organisms accounted for a largest proportion (65%), followed by Gram-negative organisms (25%), and then fungi (10%) (Wisplinghoff et al., 2004). In developed countries, the most common BSI bacteria are, *Streptococcus pneumoniae*, and *Staphylococcus aureus* (Buetti et al., 2016; Gregson and Church, 2007; Lim et al., 2014; Mehl et al., 2017). However, a few surveillance studies in pediatric or critically-ill patients identified *Klebsiella* spp. as the leading Gram-negative pathogen (Folgori et al., 2014; Orsini et al., 2012).

In hospital-acquired BSI, pathogenic agents are commonly multidrug-resistant (MDR; resistant to three or more groups of antimicrobials). Analyses from the EUROBAC study found that pathogens from HA-BSI respiratory infection were more likely to be MDR than organisms from other HA-BSI sources (76.3% vs. 56.7%, $p < 0.001$). Gram-negative organisms, mainly *Acinetobacter* and *Klebsiella* were more frequently isolated than the Gram-positive organism. The important Gram positive pathogen causing HA-BSI pneumonia was methicillin-resistant *Staphylococcus aureus* (Timsit et al., 2014).

1.6.1 Gram-positive BSI pathogens

1.6.1.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive cocci and is distributed widely in the environment. In humans, this bacterium is a common colonizer of body surfaces. *S. aureus* can cause several infections, including invasive BSI. It is typically the most common BSI Gram-positive pathogen (Deen et al., 2012; Myat et al., 2014; Nagao, 2013; Reddy et al., 2010; Son et al., 2010). Moreover, *S. aureus* is a common Gram-positive pathogen in HA-BSI. In Europe, annual incidence of BSI *S. aureus* was estimated to be 26/100,000 population/year with a 34% increase during 2002-2009 (Paulsen et al., 2015). Common primary infection sources include soft tissue infection, respiratory, arthritis, and urinary tract infections (Paulsen et al., 2015). The important resistant phenotype of *S. aureus*, methicillin resistance, has been reported around the globe (Kang and Song, 2013; Kim et al., 2014; Tariq, 2014). In a retrospective cohort study regarding nosocomial BSI in the US, *S. aureus* was number one ranked pathogen (33%) with a large proportion of methicillin-resistant *S. aureus* (MRSA) (Chen and Huang, 2014). The important source of HA-BSI due to was via intravenous catheter (Marcos et al., 2011; Tan et al., 2007; Timsit et al., 2012), and

infection with this organism was more prevalent in males than females(Buetti et al., 2016).

1.6.1.2 *Streptococci*

Streptococci are a common genus of bacteria associated with BSI. Common *Streptococcus* species causing BSI are *S. pneumoniae*, *S. pyogenes*, and *S. viridans* group(Laupland et al., 2004; Mehl et al., 2017). *S. pneumoniae* is the most well described member of the *Streptococcus* genus and the most common BSI associated with pneumoniae originating in the community. Population based studies in Europe and north America typically report *S. pneumoniae* in the list of common pathogens. The annual incidence of BSI was reported as 12-14/100,000 population in various regions(Goto and Al-Hasan, 2013; Laupland et al., 2013; Mehl et al., 2017; Skogberg et al., 2012). The rate of BSI due to *S.pneumoniae* in adult patients is between 11 and 24%(John A. Crump et al., 2011; Orsini et al., 2012; Reddy et al., 2010), which is generally lower than the rate of bacteraemia.

An important pathogenic *Streptococcus* member associated with meningitis and bloodstream infection (co-infection) in East Asia is *Streptococcus suis*. *S.suis*, a colonizer in pigs has caused large outbreaks in human in China, Hongkong, Thailand. Sepsis is the second most common clinical manifestation of *S.suis* infection after meningitis according to a systematic review in 2014(Huong et al., 2014). The proportion of *S.suis* BSI was approximately 7.6% in a tertiary hospital of Vietnam(Dat et al., 2017).

1.6.1.3 *Enterococci*

Enterococci are Gram-positive cocci, which previously belonged to *Streptococcus* group D. The incidence of BSI *enterococcus* was usually at 48/100,000 in the US (Wisplinghoff et al., 2004). The most common clinically relevant *Enterococcus* species are *Enterococcus faecalis* and *Enterococcus faecium*(Billington et al., 2014; Zheng et al., 2017). BSI associated *Enterococcus* are usually hospital-acquired. In recent years, trend of nosocomial bacteraemia *Enterococcus* has risen in many countries, infection with this organism is associated with mortality a rate of 16%-29.5%(Billington et al., 2014; Gudiol et al., 2013; Hoenigl et al., 2014; Nagao, 2013; Suppli et al., 2011). Risk factors associated with BSI *Enterococcus* include ICU admission, urinary tract infections, abdominal infections and malignancies(Henning et al., 1996; Kara et al., 2015; Nagao, 2013; Wisplinghoff et al., 2003). A big concern in *Enterococcus* is the ability of the organism to develop vancomycin resistance. Since the first description of vancomycin-resistant *Enterococcus* (VRE) in 1991, VRE has been reported as colonising organism and a pathogen internationally(Billington et al., 2014; Henning et al., 1996; Kara et al., 2015; Wisplinghoff et al., 2004). VRE is associated with treatment failure, hence leading to more death cases(Gudiol et al., 2013; Henning et al., 1996); the *Enterococci* species most commonly associated with vancomycin resistance is *Enterococcus faecium*(Gudiol et al., 2013; Wisplinghoff et al., 2004).

1.6.2 Gram-negative BSI pathogens

1.6.2.1 Salmonella

Salmonella are gram- negative bacilli that belong to the family *Enterobacteriaceae*.

Salmonella are facultative anaerobes, motile by flagella, and non-spore forming.

Salmonella are ubiquitous, and can be commonly found in soil, water and vegetation.

Salmonella can also form part the normal gut microbiota in humans and animals (Giannella, 1996).

According to the CDC, *Salmonella* is divided into two main species: *Salmonella bongori* and *Salmonella enterica*. The later is composed of six subgroups (subspecies): *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). *Salmonella bongori* itself is considered as subspecies (V)(Center for Disease Control, 2011) .

These subspecies are classified according to serology by Kauffman-White classification (JCICSP 2005)(Center for Disease Control, 2011). In this classification, each serotype is considered a unique species that results in 2,463 serotypes (or subspecies). The order of division is first by the somatic O antigen, then by flagella H antigens. In this CDC system, 59% of the described 2,463 *Salmonella* serotypes belong to *S. enterica* subsp. I, all human *Salmonella* pathogens are all in these groups. Non-typhoidal *Salmonella* (NTS) belong to the species *Salmonella enterica* and are referred to as ‘non-typhoidal’ to distinguish them from *Salmonella* Typhi and *Salmonella* Paratyphi (A, B and C), which cause enteric fever. Among these, *Salmonella enteritidis* serogroup D and *Salmonella* Typhimurium serogroup B are the two most common serotypes causing NTS infections worldwide(Centers for Disease Control and Prevention, 2014; Fields, 2006). People get infected with *Salmonella* via faecal-oral transmission. The modes of transmission are consumption of food of animal origin (eggs, poultry, undercooked meat and dairy products), fresh products that have been contaminated with animal waste, consumption of contaminated water, or direct contact with infective humans or animals(McDermott et al., 2011).

Infection with *Salmonella* has many clinical manifestations. However, there are five main types: typhoid fever (enteric fever), gastroenteritis, bacteraemia, suppurative infection and asymptomatic carrier state. Briefly, during typhoid fever, when *S. Typhi*

or *S. Paratyphi* are consumed and enters the digestive system of the host, the bacteria are engulfed by the phagocytic cells (Velge et al., 2012). These cells then deliver and present the bacteria to the macrophages in the distal ileum. The bacteria travel within the macrophages to series of lymph nodes and the reticuloendothelial tissues of the liver, spleen, and bone marrow. After reaching these sites, the bacteria multiply and pass into the bloodstream. Untreated cases can suffer from the disease for month and are at risk of severe complication such as intestinal haemorrhage or perforation (Biggs et al., 2014; Crump et al., 2015, 2004).

Typhoid is a common disease; there are an estimated 22 million cases of typhoid fever annually globally with 200,000 deaths (McDermott et al., 2011). According to a global analysis in 2008, the highest incidence of typhoid (>100 cases per 100,000 population annually) is in South Central Asia, Southeast Asia, and Southern Africa (Kothari et al., 2008). Vietnam has a medium incidence of typhoid fever and was estimated to be 23.2/100,000 per year from 1991 to 2011. Data from Ho Chi Minh City (HCMC) found that *S. Typhi* was the dominant pathogen in bloodstream infection until 2002. Since then, the number of infection has been declined steadily at a rate of approximately 30% per year (Nga et al., 2012).

Infection with NTS has four main presentations, a self-limiting gastroenteritis disease, septicaemia (the most frequent type of invasive NTS infection), suppurative infection and asymptomatic carriage (de Jong et al., 2012; Morpeth et al., 2009). The World Health Organization (WHO) reported that 93.8 million cases of enteric infection every year in the world with 155,000 fatalities annually (Ao et al., 2015). It is estimated that 2.1 to 6.5 million cases of invasive NTS (iNTS) annually, with the large proportion of cases is in Africa. Bloodstream infection, a common iNTS is often found in certain

high-risk groups such as immuno-compromised individuals, HIV patients, malaria association and malnutrition.

Enteric infections caused by NTS have similar clinical manifestations to those induced by other enteric bacteria, and can rarely be distinguished by clinical presentation. NTS-associated gastroenteritis is characterised by abdominal pain, fever, watery diarrhoea, and occasionally mucoid or bloody diarrhoea. Vomiting and/or nausea occur frequently but are not severe or protracted. The disease is usually self-limiting but can be more severe in the very young and elderly or immunocompromised, with a typical duration of illness of 4 to 7 days (Gordon, 2011). In severe illnesses, antimicrobials should be prescribed. There is no effective vaccine against NTS; hence, disease prevention and control programs mostly rely on improving personal hygiene and sanitation with particular attention paid to food safety.

There are very few studies regarding invasive NTS infection in Asia, therefore data describing the clinical symptoms of this disease mostly originates from Africa. The common presentation is general bacteraemia, with high fever and splenomegaly. Meningitis is not as common as sepsis but NTS had been reported as a second most cause in bacterial meningitis in Malawi. The fatality rate in NTS meningitis is very high in both children and adults (Crump et al., 2015; Feasey et al., 2012).

The epidemiology of invasive NTS is highly geographical and common in parts of sub-Saharan Africa. A systematic literature review in 2015 estimated that approximately 3.4 million cases of iNTS with more than 600,000 deaths annually in the world, with the highest incidence in African countries (227/100,000 population). (Ao et al., 2015; Crump and Heyderman, 2015). Over the past 10-15 years invasive NTS has come to forefront of *Salmonella* research, lead mainly by groups in Kenya, Malawi, and

Tanzania (Arthur et al., 2001; John A. Crump et al., 2011; Melita A. Gordon et al., 2002). It appears that invasive NTS disease is associated with a regional epidemic in sub-Saharan Africa and is associated with HIV and Malaria. The disease appears to affect malnourished or malaria associated children, and can have a mortality rate as high as 40% in some locations (Gordon, 2011). Additionally, a systematic review on blood culture positive patients in Africa showed that NTS accounted for 17% of 5,578 positive blood samples and was associated with HIV infection; *S. Typhi* were isolated at a lower rate (9.9%) and mostly in non-HIV infected individuals. *S. Typhimurium* and *S. Enteritidis* are the two most common serovars associated with invasive NTS disease (Gordon, 2011). Recently, a multidrug-resistant strain, *S. Typhimurium* ST313, has been identified as the predominant strain of iNTS in Africa which contributed to the burden of disease in this region (Crump and Heyderman, 2015).

The limited data available on invasive NTS diseases in Asia originates from a multi-centre study conducted in people aged 2-15 years, which found that NTS accounted for <1% (only 6 cases/ 20,537 total blood samples) of all the pathogens associated with febrile disease (Khan et al., 2010). This study did not collect data on HIV or malaria co-infection but the incidence of the two diseases is not as high as in parts of sub-Saharan Africa.

1.6.2.2 *E. coli*

In most population-based studies, *E. coli* has been found to be the predominant pathogen in BSI (Buetti et al., 2016; Lim et al., 2014; Son et al., 2010). *E. coli* is a common pathogen for both CA-BSI and HA-BSI, especially in elderly patients (Yahav et al., 2016). The proportion of BSI due to *E. coli* varies from 28% -59.4% in all agents of BSI (Buetti et al., 2016; Lim et al., 2014; Son et al., 2010). Longitudinal studies have

generally shown that the trend of *E. coli* BSI increases overtime with more and more MDR organisms isolated (Datta et al., 2012; Lim et al., 2014). European longitudinal studies demonstrated that BSI caused by *E. coli* were found more commonly in females than males (Norway: 93 vs. 56/100,000 person-years, Switzerland 36% vs. 23%; $p < 0.001$) (Buetti et al., 2016; Mehl et al., 2017). *E. coli* was also more prevalent in people over 65 years old (Buetti et al., 2016). Season also appears to have some influence on the incidence of *E. coli* BSI. A ten-year study of seasonal variation in the US concluded that the incidence of *E. coli* BSI increased in the warmer seasons. With every 5.5°C increased, the bloodstream incidence of *E. coli* increased >7% ($p = 0.004$) (Al-Hasan et al., 2009).

1.6.2.3 *Klebsiella*

In many BSI studies, *Klebsiella pneumoniae* is the second most commonly isolated pathogen after *E. coli* (Kang et al., 2005; Koupetori et al., 2014; Son et al., 2010; Vlieghe et al., 2013). *K. pneumoniae* is often characterized by the ability of broad-spectrum resistance to antimicrobials, producing not only extended-spectrum beta lactamases (ESBL) but also carbapenemases (Orsini et al., 2012; Tan et al., 2007). Highly-resistant *K. pneumoniae* are most commonly isolated from the hospital, especially ICU settings (Arnoni et al., 2007; Marra et al., 2011; Orsini et al., 2012; Tan et al., 2007). In a recent study, ESBL-producing *K. pneumoniae* was also associated with community-acquired infection. (Lee et al., 2011)

1.6.2.4 Other significant Gram-negative pathogens

Acinetobacter baumannii and *Pseudomonas aeruginosa* are both important Gram-negative nosocomial pathogens. While *Klebsiella* spp. are associated with both

community and hospital-acquired infections, *A. baumannii* and *P. aeruginosa* are more commonly associated with hospital-acquired infection (Wisplinghoff et al., 2004). They are characterized with causing severe nosocomial infections and are commonly have an MDR phenotype (Al-Mously, 2013; Mudau et al., 2013; Wisplinghoff et al., 2000; Zavascki et al., 2010). *Acinetobacter* species are Gram-negative coccobacilli, which are widely distributed in soil, water, and able to colonise in hospital environment or plastic. In human, they are able to colonize the external and internal surfaces of healthy people and patients (Thom et al., 2010). This pathogen also has the ability to acquire antimicrobial resistant genes making it resistant to many broad-spectrum antimicrobials (Zavascki et al., 2010). *Pseudomonas aeruginosa* is an aerobic Gram-negative bacillus, which is well known for causing infection in burn, ventilator-associated pneumoniae, urinary tract infection, skin and soft tissue infection and neutropenic sepsis (Micek et al., 2005; Mudau et al., 2013; Wisplinghoff et al., 2004). Its specific characteristics are an ability to cause a severe systemic infection and a propensity to develop MDR (Arnoni et al., 2007; Zavascki et al., 2010).

1.7 Contamination

During the process of blood culturing, environment bacteria can access the blood sample and yield positive result after incubation. This contamination can lead to unnecessary antimicrobial treatment or further laboratory analysis for repeat testing. The general rate of blood culture contamination is expected to be <3% (Bentley et al., 2016; Clinical and Laboratory Standards Institute, 2007), recent studies have reported contamination rates within the range of 4%-14% (Bentley et al., 2016; Obeng-Nkrumah et al., 2016; Self et al., 2013; Vlieghe et al., 2013). Factors associated with contamination include critical illness, heavy workload in a healthcare setting, blood collection via intravenous catheter (Chang et al., 2015; Lloyd Towns et al., 2010).

Blood culture contamination is defined if one blood culture bottle yielding a possible contaminating bacteria, which includes coagulase negative *Staphylococcus*, *Corynebacterium spp.*, *Bacillus spp.*, *Diphtheroid spp.*, *Micrococcus spp.*, *Propionibacterium spp.*, and *Streptococcus viridans* group (Gregson and Church, 2007; Kanoksil et al., 2013; Kiertiburanakul et al., 2012). However, a large investigation of blood culture contamination found that some bacteraemia associated pathogens such as *Staphylococcus aureus* can be contaminants (Chang et al., 2015). If a set of two bottles are collected simultaneously, it is possible to rule out the contamination based on Clinical Laboratory Standard Institute (CLSI) blood culture guidelines (Clinical and Laboratory Standards Institute, 2007). However, in case only one blood culture is taken, discussion between microbiology laboratory and clinicians is required to assess a plausible association.

1.8 Laboratory diagnosis of BSI

1.8.1 Blood culture

According to the CLSI, a blood culture is a laboratory test to detect the presence or bacteria or fungi in the blood by using a nutrient media (Clinical and Laboratory Standards Institute, 2007). Therefore, a blood culture is an obligate laboratory test to diagnose a BSI. The aim of the test is to grow and identify bacteria present in the patient's blood. Moreover, the isolated bacteria can be tested for using antibiotic susceptibility, which helps select an appropriate antimicrobial regime for treatment.

To remove the colonizing skin bacteria and prevent commensal organism from contaminating the blood culture skin antisepsis is performed before a blood sample is taken. It is an essential first step for the blood culture technique. Common skin antiseptic substances include alcohol, povidone-iodine, and chlorhexidine. Alcohol or

alcohol in combination with other antiseptics has been found to be most effective disinfectants with the lowest contamination rate (Caldeira et al., 2011; Calfee and Farr, 2002; Kiyoyama et al., 2009).

According to CLSI guidelines for the management of severe sepsis and septic shock, at least a set of one aerobic culture and one anaerobic culture should be taken for culturing (Clinical and Laboratory Standards Institute, 2007; P. Dellinger et al., 2013; Towns et al., 2010). However, some studies showed that the positivity rate was approximately 80-93% of BSI cases if a set was used. There have been suggestion to take more (up to four) consecutive sets of blood cultures for optimal pathogen discovery (Cockerill et al., 2004; Kirn and Weinstein, 2013; Lee et al., 2007). Instruction for infective endocarditis should include three sets of blood culture within one hour (Clinical and Laboratory Standards Institute, 2007; Cockerill et al., 2004). In case only one blood vial is taken, aerobic culture is advised. The number of blood samples does directly affect the recovery rate of pathogen. Many studies showed that the more samples collected, the higher chance to isolate the causative pathogen (Kirn and Weinstein, 2013; Lee et al., 2007; Weinstein et al., 1994).

In adults, the number of organisms circulating in the blood is typically <1 CFU/ml. Therefore, the possibility of yielding blood culture is strongly associated with the amount of blood volume inoculated into a culture bottle (Weinstein, 1996). A standard amount of blood is 10ml inoculated into a culture bottle (P. Dellinger et al., 2013). An adequate blood volume collection plus 2 sets of cultures improved the pathogen discovery rate to up to 90-95% of bacteremias (Towns et al., 2010). In paediatrics, there is higher concentration of pathogen organisms in the children's blood than in adults, therefore an adequate volume is 1-5ml of blood depending on body weight and age (Kirn and Weinstein, 2013; Weinstein, 1996).

There are two common types of blood culture systems: a traditional manual system and a modern automatic system. The traditional manual blood culture system uses a broth-based approach (agar broth biphasic blood culture) in which blood is infused with the broth bottle before incubation. Daily macroscopic examination is required to observe the signs of organism growth such as turbidity, colony, and haemolysis. The organisms are then subcultured onto agar plates for further testing. All broth bottles without obvious signs of bacterial growth sign are required to be subcultured after 7-10 days incubation(Doern, 1994; William Dreyer, 2012). The conventional blood culture technique has some disadvantages including, false positives and false negatives, limited ability to grow anaerobes and fastidious bacteria, and more labour (William Dreyer, 2012). Manual culture is however low cost and suitable and easy to run and justify in smaller laboratories.

Overcoming many of the weaknesses of conventional blood culture system, automated blood culture systems have been developed and rapidly applied in clinical laboratories from the 1970s. The automated blood culture system is considered to have had a major impact in the field of clinical microbiology. The principle of an automated blood culture system is the indirect detection of organism's presence based on the concentration of CO₂ level. In general, if an organism is replicating in the culture media it will utilize the available carbon source and release CO₂. Recent commercial automated blood culture such as BACTEC, BacAlert, or VersaTrek have used this principle to develop their own bacterial detection systems(Clinical and Laboratory Standards Institute, 2007; Cockerill et al., 2004; Dilnessa et al., 2016; Gebert et al., 2008). If the concentration of CO₂ reaches the detection limit, the machine alerts the operator and the bottle requires subculture, further identification, and antimicrobial susceptibility testing. All the bottles require incubation for a minimum duration of 5 days, and then discharge as negative

results. In some circumstances, blood culture can be incubated for a longer periods to identify slow-growing organisms such as HACEK group, *Brucella*, or fungi(Kirn and Weinstein, 2013; Weinstein, 1996). However, there are some undeniable issues about automatic systems such as cost, power requirement, and maintenance.(Gebert et al., 2008; Lloyd Towns et al., 2010; William Dreyer, 2012)

Several variables can impact on the ability to isolate organisms from a blood culture. Prior antimicrobial consumption is a major inhibitor of the growth of bacteria in blood culture. Therefore, the addition of substances that neutralise antimicrobials is essential for enhancing bacterial growth. The most popular antimicrobial removal agent is resin, which was firstly introduced into the BACTEC system blood culture bottle (Becton Dickinson). Resin-containing media have been proven to eliminate most common antimicrobials such as: cephalosporins, carbapenems, fluoroquinolones, aminoglycosides, and vancomycin.(Crepin et al., 1993; Rohner et al., 1997) A comparison between resin-containing media and non-resin-containing media showed that higher recovery rate was found in resin-containing media.(Cockerill et al., 1997) Guidelines recommend the use of resin-containing bottles in sepsis management(Towns et al., 2010).

Whenever an incubated blood culture has positive signal, it needs to be removed from the incubator for further testing. A certain amount from the blood culture is withdrawn for direct Gram staining. The primary morphology of bacteria on Gram stain is an important indicator that helps guide antimicrobial treatment. The type of media for isolation will be selected depending on the characteristics of the organism on staining. Typically, blood agar, chocolate, agar and MacConkey agar are suitable for most bacteria; Sabouraud agar is used for fungal growth. Routinely, subculture media will be incubated at 37°C for up to 5 days with everyday examination.

1.8.2 Identification of BSI bacteria

1.8.2.1 Biochemical tests

Bacteria have different metabolic profile, which can be used to differentiate organisms from the other bacteria. Therefore, a series of biochemical test have been used to identify specific metabolic products. Table 1 shows common biochemical tests in clinical microbiology laboratories(Giannella, 1996; Koneman Elmer, 1992; Macffaddin F. Jean, 1976; Patricia Clarke and S.T. Cowan, 1952):

However, bacteria in a same species (e.g. *Enterobacter*, *Pseudomonas*, *Streptococcus*) may share many similar biochemical reactions. Hence, the more biochemical reactions performed, the more precise the identification. Before the availability of API strip testing, clinical laboratories used a limited number of manual biochemical tests for bacterial identification, hence limited the accuracy of identification. Currently, modern identification systems (API, VITEK system, and MALDI- TOF) are available and have made a great impact on clinical microbiology.

Table 1. 1 Common biochemical tests for bacteria identification

Name of test	Purpose	Common application
Catalase	Detect the ability to catalyse the release of oxygen from H ₂ O ₂	Differentiate between <i>Staphylococci</i> (catalase positive) and <i>Streptococci</i> (catalase negative)
Coagulase	Detect the coagulase enzyme that converts fibrinogen (soluble) in plasma to fibrin (insoluble).	Differentiate between (coagulase positive) and other <i>Staphylococci</i> (coagulase negative)
Bile solubility	Lyses bacteria cells in the presence of bile	Differentiate between (possible soluble) and other <i>Streptococci</i> (non-soluble).
Oxidase	Detect the ability to produce cytochrome oxidase	Supportive identification of <i>Pseudomonads</i> , <i>Neisseria</i> , <i>Moraxella</i> , <i>Pasteurella</i> sp.
Carbohydrate fermentation	Detect acid products from the fermenting specific carbohydrate (glucose, lactose, sucrose...). Changing colour of pH indicator indicates the acid production.	Differentiate among bacterial groups (Exp: Most Enterobacteriaceae members are glucose fermenter).
Urease	Detect urease enzyme that hydrolyses urea to ammonia and carbon dioxide	Supportive identification of <i>Proteus species</i> , <i>Morganella species</i> , <i>Providencia species</i>
Citrate	Detect the ability to use citrate as a source of nitrogen, which breaks down citrate to oxaloacetate and acetate.	Differentiate between Enterobacteriaceae members (Exp: <i>Salmonella</i> has citrate test positive, has citrate test negative)
Indole	Detect the ability to produce indole by deamination of tryptophan	Differentiate between Enterobacteriaceae members (Exp: <i>E.coli</i> has indole test positive, <i>Klebsiella pneumoniae</i> has indole test negative)
Methyl red	Detect the ability to use glucose and convert it to stable acids (acid lactic..)	Differentiate between Enterobacteriaceae members (Exp: <i>E.coli</i> has methyl red test positive, <i>Klebsiella pneumoniae</i> has methyl red test negative)

1.8.2.2 Matrix assisted laser absorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS is a major advance for clinical microbiology for fast and accurate organism identification. The principle of MALDI-TOF MS is that bacterial cells are fragmented and the components are accelerated in an electric field, then quantified according to their mass-to-charge value and analysed by a mass spectrometer. Every bacterial genus/species has a distinctive spectra which can be compared with a database software so that the organism with the nearest spectra can be identified (Murray, 2012; N. Singhal et al., 2015). An important application of MALDI-TOF is the possibility to identify bacteria directly from the alerted blood culture samples. Therefore, a confirmed identification is generated within an hour instead of waiting 18-24 hours for the growth of colonies. However, the identification process directly on blood cultures requires more steps and reagents than on the colonies (Murray, 2012). Advantages of MALDI techniques are simplicity, rapid, high accuracy, and low sample volume requirement, also cheap to run, which overcomes all the disadvantages of biochemical identification tests (Pavlovic et al., 2013). However, MALDI-TOF MS is unable to give identification if the bacterial spectrum is not yet in the database and cannot identify between very closely related organisms such as *E. coli* and *Shigella*.

1.8.2.3 Molecular identification

Besides MALDI-TOF, molecular-based assays are a promising tool to diagnose rapidly and precisely the cause of BSI. In recent years, many studies have emphasized the usefulness of PCR in for identifying pathogens associated with BSI. Molecular identification from culture has proven its value for detecting most pathogens, even rare

organism. The most common target gene is the 16S rRNA gene, which is present in all bacteria, and possesses a conserved specific genetic section. This target gene is sequenced and compared with the gene reference database libraries (MicroSeq, Genbank, Ribosomal Database Project). Acceptable identity score is equal or more than 97% for the bacterial genus and 99% for the species. However, if the group of bacteria share >99% identical 16S rRNA sequence, they are unable to be differentiated by this method.

Another application of 16S rRNA sequencing on direct blood is for rapid detection of pathogen within hours. Based on this technique, commercial test kits have been developed. SeptiFast (Roche) is multiplex PCR targeting the ribosomal gene, and has the ability detect 20 common pathogens causing BSI. The performance of the SeptiFast test has been evaluated with high sensitivity and specificity with a turnaround time of 4-6 hours (Dark et al., 2011; Rutanga and Nyirahabimana, 2016). Another commercial test kit, IRIDICA (Abbot), which can detect bacteria, fungi, and some antimicrobial resistant genes, had an overall agreement of up to 70% with blood culture. The IRIDICA test discovered more bacteria and fungi than blood culture. Moreover, the blood volume required for PCR is six times smaller than that for the blood culture techniques (5 ml vs. 30 ml) and the time to result was also shorter (Jordana-Lluch et al., 2015). A systematic review about the clinical impact of PCR for BSI showed that all PCR assays delivered rapid result less than 6 hours which is very crucial for sepsis management.

1.8.2.4 Genotypic bacterial typing methods

Genotypic typing (genotyping) is a process to determine the genetic variant (specific alleles or single nucleotide polymorphisms) of an individual bacterium by polymerase

chain reaction (PCR) amplification and sequencing. The main purpose of genotyping is epidemiological surveillance(Tümmler, 2014).

There are several methods for bacterial genotyping, but pulsed-field gel electrophoresis (PFGE) and Multilocus Sequence Typing (MLST) are the most commonly used(Tümmler, 2014). PFGE is a laboratory technique that allows different sizes of DNA fragments from 10kb to 10Mb to be separated by using electrophoresis. Although PFGE is expensive and laborious, it is still the gold standard typing method for investigation of bacterial pathogen and outbreaks in many public health laboratories (Herschleb et al., 2007; Peacock et al., 2002).

MLST is a technique that sequences fragments of housekeeping genes (typically 7) to identify genetic variation with an individual isolate from a known bacterial species. The purpose to identify distinct allelic profiles (sequence type (ST)) in the housekeeping genes(Ibarz Pavón et al., 2009; Tümmler, 2014). The sequence data library for MLST (MLST databases: <http://www.mlst.net/databases/>) are available online for comparison and surveillance. The MLST technique is highly reproducible between laboratories, can be performed on living or dead cells, or even of clinical specimen (e.g. blood, cerebrospinal fluid)(Ibarz Pavón et al., 2009). Due to its high value in epidemiology surveillance, MLST has been applied to study several bacterial epidemics such as meningococcal disease, outbreaks of typhoid, MRSA, and MDR *Pseudomonas aeruginosa*.(Baker et al., 2011; Brehony et al., 2007; Faria et al., 2008; Mudau et al., 2013)

1.8.2.5 Whole genome sequencing

Whilst ribosomal PCR, PFGE, and MLST are targeted towards specific DNA, whole genome sequence explores the entire whole DNA content of an individual bacterium. Therefore, whole genome sequencing provides more information about the genetic composition of the organism than other genotyping methods. However, whole genome sequencing can be complicated by several processes (technical ability, cost, bioinformatics analysis data interpretation) As a result, whole genome sequencing is currently more suitable for research purposes than routine clinical use(Khromykh and Solomon, 2015; Salipante et al., 2015).

Overall, molecular-based techniques have many advantages in a diagnostic microbiology laboratory for BSI. Nevertheless, these methods are not performed routinely, particular in low income countries, due to high cost and extensive skill required to perform these techniques(Angeletti et al., 2015). Therefore, blood culture remains the gold standard for laboratory diagnosis of BSI in clinical settings globally.

1.8.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is a laboratory method to obtain the susceptibility of an isolated organism to one or many antimicrobials. The result of the AST provides vital information about antimicrobial choice and treatment regime for an individual infection. As BSI is a critical disease, some types of AST are able identify some specific resistant mechanisms (e.g. ESBL, KPC) or phenotype (MRSA) so that appropriate antimicrobials can be administered in an appropriate clinical time frame.

Although there are many technical methods for AST, the results should be reported in a standardised fashion, the tested pathogen is reported as susceptible/intermediate/ or resistant (Susceptible, the antimicrobial agent is able to treat the infection caused by the

isolated organism; Intermediate, the antimicrobial agent is able to treat the infection if a higher dose is used or higher concentration in infected sites is achieved; and Resistant; the antimicrobial agent is unable to treat the infection caused by the isolated organism.

The functionality of antimicrobials is further assessed against an organism by its Minimum inhibition concentration (MIC) ($\mu\text{g/ml}$). The MIC is defined as the lowest concentration of antimicrobial that inhibits visual growth of bacteria in a defined period of time. An MIC is obtained under *in-vitro* testing conditions. The broth dilution test is considered to be the gold standard method for antimicrobial susceptibility testing. This technique requires test tubes containing a defined concentration of bacteria ($1-5 \times 10^5$ CFU) in liquid growth medium. A series of two-fold dilution of the screened antimicrobial (e.g. 1, 2, 4, 8, and 16 $\mu\text{g/ml}$) are dispensed in the test tubes and incubated for 18-24 hours at 35°C. After incubation, the tubes are examined for the turbidity, which indicates growth of bacteria. The lowest concentration of antimicrobial that has no evidence of bacterial growth is the MIC of the antimicrobial. The MIC is compared with the interpretation range in published guidelines such as those validated by CLSI and EUCAST (European Committee on Antimicrobial Susceptibility Testing). The main advantage of broth dilution is to give a quantitative result (MIC value), which is more precise than the qualitative result generated by other less quantitative methods. However, the broth dilution method is laborious and utilises differing reagents (Amsler et al., 2010; Jorgensen et al., 2009; Mounyr Balouiri n and Moulay Sadiki, 2015). Therefore, this method is generally used more for research than for clinical purpose.

1.8.3.1 Antimicrobial gradient method

The antimicrobial gradient method uses an antimicrobial gradient plastic strip to test on an agar plate. The plastic strip contains an increasing gradient antimicrobial

concentration, which is impregnated into the strip and called an E-test. After incubation the MIC result can be read at the point of intersection between the bacterial growth and the value printed on the E-test strip. The gradient method gives quantitative result similar to the agar dilution method but is easier to perform. Many comparative studies have shown that the E-test method produces a good correlation with the broth dilution method (Amsler et al., 2010; Heijden et al., 2007; Mounyr Balouiri n and Moulay Sadiki, 2015). E-tests are suitable for obtaining the MIC of one or few antimicrobials because the price for each test strip is costly.

1.8.3.2 Disk diffusion test

Disk diffusion test is a manual antimicrobial susceptibility test, which was standardized by Kirby and Bauer in 1960s. The disk diffusion method is continuously updated by the CLSI annually for new resistance mechanisms and interpreted for newer antimicrobials (Clinical and Laboratory Standards Institute, 2015; Hudzicki, 2009). Disk diffusion is an alternative method to the broth dilution technique. In the Kirby-Bauer method, a specific bacterial suspension (~0.5 McFarland) is applied on a 90 mm-diameter Muller-Hinton agar plate. The paper disk, which is impregnated with a known concentration of an antimicrobial, is placed on the prepared Muller-Hinton agar. The plate is incubated in 37°C for 16-24 hours in aerobic environment or in 5% CO₂ depending on the bacteria species. After a sufficient incubation time, the plate can be examined for the inhibition zones around the antimicrobial disks. The diameter of inhibition zone is measured in millimetres and compared with the criteria of the CLSI or other guidelines. The result of each antimicrobial will be described as susceptible, intermediate, or resistant to the tested bacteria. The Kirby-Bauer method is very popular in clinical microbiology laboratory due to its simplicity and standardisation (Hudzicki, 2009; Jorgensen et al., 2009). However, the antibiogram generated by disk diffusion

method is not precise for slow-growing or rare organism, which have not yet been standardized by guidelines(Gaudreau et al., 2008; Hudzicki, 2009).

1.8.3.3 Automatic systems for antimicrobial susceptibility testing

The limitation of manual antimicrobial susceptibility testing are that they are laborious and have a long turn-a-around time. Automatic blood culture systems have been developed to overcome these limits. Common automatic susceptibility testing systems are VITEK (BioMerieux), Phoenix (Becton Dickinson), and Microscan Walkaway (Siemens). Each system has a private card (VITEK)/panel (Phoenix)/tray (Walkaway) that contains multiple antimicrobials in different dilutions. All automated systems generate MICs for several antimicrobials simultaneously. These systems use colorimetric or turbidometric detection for the growth of bacteria in antimicrobial dilution wells within 6-14 hours. Moreover, these systems have software, which can detect specific resistance activity (ESBL, AmpC, MRSA, VRE, and KPC).

A comparison between various automated blood culture systems and manual testing methods generally show high agreement. A study in India compared the antimicrobial susceptibility results of common pathogens by the Phoenix (Becton Dickinson) and disk diffusion method. Categorical agreement in this study was as high as 98% and 95.7% for Gram-negative and Gram-positive bacteria, respectively(Duggal et al., 2012).

Automated systems also generated good results for specific antimicrobials such as vancomycin and colistin. A comparative study of many antimicrobial testing methods for *Acinetobacter baumannii* revealed that the VITEK and E-test method had good interpretable agreement for colistin susceptibility with broth dilution, but not disk diffusion(Lo-Ten-Foe et al., 2007).

1.8.4 Common resistant mechanism

1.8.4.1 Extended-spectrum- β lactamases (ESBLs)

The Extended-spectrum- β lactamases (ESBLs) are a group of enzymes that hydrolyse third and fourth generation cephalosporins and monobactam but have no activity against cephamycin (cefoxitin) and carbapenems. The ESBLs are enzymes encoded by various plasmid encoded resistance genes (TEM, SHV or CTX-M), which may be able to transfer across different Gram-negative bacterial species.

The combination disc method is a confirmatory method for ESBL activity and is recommended by the CLSI, CDC, and BSAC (British Society for Antimicrobial Chemotherapy) guidelines (British Society for Antimicrobial Chemotherapy, 2012; Clinical and Laboratory Standards Institute, 2015; “Laboratory Detection of Extended-Spectrum β -Lactamases (ESBLs) | HAI | CDC,” 2010). This method should be used routinely for all clinical, *Klebsiella* and *Proteus* due to the high proportion of ESBLs positive organisms within these species. This method incorporates a 3rd generation cephalosporin (mostly cefotaxime or ceftazidime) alone and in combination with clavulanic acid under disc diffusion test conditions. After incubation at 35°C overnight, ESBL activity is confirmed if the diameter of 3rd cephalosporin-clavulanic acid inhibition zone >5mm larger than that of the 3rd cephalosporin alone.

The Double- disc method is an alternative screening method for ESBLs detection and is performed under disc diffusion conditions. A ceftazidime/cefotaxime disc and an amoxicillin-acid clavulanic disc are placed 25-30 mm apart, centre-to-centre. After incubation at 35°C overnight, ESBL activity is considered if there is a sign of an increasing zone of inhibition from amoxicillin-acid clavulanic toward the 3rd cephalosporin disc. The distinctive image of this method is called as “champagne-cork”, or the “key-hole”. (British Society for Antimicrobial Chemotherapy, 2012; Overdevest et al., 2011).

The E-test designated specifically for ESBL detection contains gradients of cefotaxime or ceftazidime at one end and combination of cefotaxime or ceftazidime and clavulanic acid at the other. ESBL activity is confirmed if the MIC of combination is 3 times lower than the MIC of single antimicrobial (British Society for Antimicrobial Chemotherapy, 2012; “Laboratory Detection of Extended-Spectrum β -Lactamases (ESBLs) | HAI | CDC,” 2010).

1.8.4.2 AmpC lactamases

AmpC lactamases are an alternative β -lactamase enzyme that can be encoded by either chromosomal or plasmid associated genes. Chromosomal AmpC lactamases are present in some members of Enterobacteriaceae such as *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., *Aeromonas* spp., and *Morganella* spp. Plasmid mediated AmpC lactamases are commonly found in *Klebsiella* species. Organisms that had have AmpC lactamase activity are resistant against penicillins, 3rd cephalosporins (ceftazidime, ceftriaxone, cephamycin) and monobactams but susceptible to 4th cephalosporins (cefepime) and carbapenems (G. A. Jacoby, 2009; Mohd Khari et al., 2016). Unlike ESBLs, there is currently no standard manual testing method for AmpC lactamase detection. However, there are some recommended detection methods such as the ceftazidime disk screening test, the ceftazidime-cloxacillin double disk synergy test, the AmpC induction test, and the D69C AmpC detection set. (Mohd Khari et al., 2016; Polsfuss et al., 2011; Tan et al., 2009)

Ceftazidime disk screening test

In the disk diffusion test, AmpC lactamase production is suspected if the bacteria resistant to cefoxitin and 3rd cephalosporins and susceptible to 4th cephalosporins. The sensitivity of using cefoxitin as a screening agent was 97% while the specificity was 78.7% (Polsfuss et al., 2011).

Cefoxitin-cloxacillin double disk synergy test

In the disk diffusion test, a cefoxitin disk (30µg) and a combination disk of cefoxitin-cloxacillin (30µg-200µg) are placed together. After overnight incubation, if an inhibition zone of the combination disk is equal or >4 mm greater than that of the cefoxitin disc, AmpC lactamase production is confirmed. The sensitivity and specificity of this method is around 95% (Tan et al., 2009).

AmpC induction test

This method is used to detect induced AmpC lactamase activity. In some bacteria, the activity of AmpC lactamase will be expressed in the presence of strong antimicrobial inducer, such as cefoxitin or imipenem. During the disk diffusion test, a 3rd cephalosporin is placed near cefoxitin/imipenem. A flattening zone of 3rd cephalosporin toward the inducer indicates the inducible AmpC lactamase. This method had low sensitivity (25%) and high specificity (99%) (Tan et al., 2009).

1.8.4.3 Carbapenem-resistant *Enterobacteriaceae* (CRE)

During the routine antimicrobial susceptibility testing, organisms that are non-susceptible to any carbapenems (ertapenem, imipenem, meropenem) are indicative of carbapenemase production. CRE is confirmed later by a carbapenemase test. However, there are many types of carbapenemase enzymes, such as *Klebsiella pneumoniae* carbapenemase (KPC), OXA enzymes, the Metallo-β-lactamase (MBLs) group

(comprised of IMP, VIM and NDM enzymes), but there is no perfect test to detect all enzyme types. Carbapenemase tests include, the broth micro dilution MBL screen, gradient MIC strip, Modified Hodge test, Carba NP test, the Carbapenemase inactivation method, MALDI-TOF MS, PCR, and whole genome sequencing(Kempf et al., 2012; Kost et al., 2017; Lutgring and Limbago, 2016; Miller and Humphries, 2016; Tamma et al., 2016). The Modified Hodge's test, the Carba NP test, and PCR are recommended by the CLSI guidelines (Clinical and Laboratory Standards Institute, 2015; Lutgring and Limbago, 2016).

1.8.4.4 Methicillin-resistant *Staphylococcus aureus* (MRSA)

With the high percentage of infections associated with MRSA the CLSI proposed the routine testing of MRSA from all *Staphylococcus aureus* isolates (Clinical and Laboratory Standards Institute, 2013). The detection methods for MRSA include, the cefoxitin disk-screening test, oxacillin disk screening test, latex agglutination test for PBP2a, and PCR for the *mecA* gene. The oxacillin disk screening test has 80% sensitivity and 100% specificity, the other tests have 100% sensitivity and specificity (Marlowe and Bankowski, 2011; Pourmand et al., 2014).

1.8.4.5 Vancomycin resistant enterococcus (VRE)

Screening method for VRE utilises a bile esculin agar plate containing vancomycin or a vancomycin E-test(Clinical and Laboratory Standards Institute, 2015). A confirmatory PCR test detects the *vanA*, *vanB*, or *vanC* gene from enterococcus isolates or directly from clinical samples(Mak et al., 2009; Özsoy and İlki, 2017; Young Seo et al., 2011).

1.9 Treatment of BSI

The more severe the sepsis becomes, the higher greater the risk of death; fatality can exceed 50% from severe sepsis and 80% from septic shock(Martin, 2012). Therefore, it is crucial to start treatment as soon as possible and antimicrobial are one of the key elements for managing BSI. According to the Guidelines for sepsis management, antimicrobials should be administered within one hour after a diagnosis of sepsis has been made(R. Dellinger et al., 2013). Any delay on antimicrobial prescription or inappropriate initial antimicrobial treatment for BSI have been shown to be associated with prolonged hospitalization, high treatment costs, and high mortality. A study regarding *Pseudomonas aeruginosa* associated BSI in the US concluded that delaying appropriate antimicrobial therapy significantly increased the mortality by an odd ration of 4.1, ($p=0.03$). It has also been reported that MDR *P. aeruginosa* represents an independent risk factor for late appropriate antimicrobial therapy (OR 4.6, $p<0.001$)(Lodise et al., 2007). A further study of BSI with Gram-negative bacilli in Korea showed that inappropriate antimicrobial treatment was found in 52.8% patients which lead to a 38% increase in mortality rate(Kang et al., 2005).

The duration of antimicrobial usage in BSI remains under debate. The treatment of BSI can include a short course (5-7 days) or long course (10-14 days). However, according to a meta-analysis review, short course antimicrobial treatment is as effective as long course for BSI(Havey et al., 2011). These data support the reducing the use of antimicrobials, which decreases cost, adverse event, and the generation of resistant organisms.

Appropriate antimicrobial therapy in BSI is defined as the antimicrobial in clinical use has *in vitro* activity against BSI pathogen and the route of administration provides enough bioavailability for treatment. It is generally recommended that empirical

antimicrobial treatment should use the intravenous broad-spectrum antimicrobials. The choice of antimicrobial should depend on infection site (e.g. pneumoniae, and abscess) and source (e.g. hospital, community origin), and using local knowledge of susceptibility patterns of common BSI pathogens. After the antibiogram is reported, escalation to a narrow antimicrobial should be considered for every BSI case to reduce the risk of resistance development (R. Dellinger et al., 2013; Timsit et al., 2014).

1.10 Antimicrobial resistance: an increasing global health problem

Antimicrobial resistance has been identified as a major threat in global health. Broad-spectrum antimicrobial resistance such as ESBL and MRSA are now common in community and hospital-acquired BSI (Kang et al., 2012; Kim et al., 2014; Paulsen et al., 2015; Serefhanoglu et al., 2009; Tariq, 2014). In many ICUs, MDR organisms, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the most common pathogens for hospital-acquired BSI (Micek et al., 2005; Mudau et al., 2013; Tjoa et al., 2013; Wisplinghoff et al., 2000; Zavascki et al., 2010). Realizing the importance of antimicrobial resistance, The European Centre for Disease Prevention and Control (ECDC) and the Centres for Disease Control and Prevention (CDC) have established definitions for several levels of antimicrobial resistance based on the antimicrobial testing lists from CLSI and EUCAST guidelines (Magiorakos et al., 2012). There are currently three level of antimicrobial resistance, Multidrug-resistant (MDR; non-susceptibility to at least one agent in ≥ 3 antimicrobial categories), Extensively-drug-resistant (XDR; non-susceptibility to at least one agent in all but still remains susceptible to one or two antimicrobial categories), and Pan-drug resistant (PDR; non-susceptible to any antimicrobial in all antimicrobial categories).

1.10.1 Current status of antimicrobial resistance (AMR) in Asia

Antimicrobial resistance has been identified as a major threat recently, especially in the Asia. All the most infamous antimicrobial resistant organisms, including MRSA, VRE, and CRE are found circulating in many Asian countries (Ahn et al., 2001; Kang and Song, 2013; Kim et al., 2014; Manchanda et al., 2010; Phetsouvanh et al., 2006). According to Asian Network for Surveillance of Resistant Pathogen (ANSORP), MRSA accounted for 25% and 67% of all community and hospital- acquired infections caused by *S.aureus*, respectively (Kang and Song, 2013). The Study for Monitoring Antimicrobial Resistance Trends (SMART) found that prevalence of ESBL organisms in Asia remained particularly high in comparison with other parts of the world, increasing from 18% to 40% of defined organisms within 10 years (Morrissey et al., 2013). The predominant type of ESBL type in Asia is CTX-M15. Moreover, AmpC lactamases were found to coexist with ESBL genes, and AmpC lactamase genes were found in >60% of ESBL expressing isolates (Sheng et al., 2013).

1.10.2 Vietnam

Vietnam, is located Southeast Asia, and has a high reported rate of antimicrobial resistant organisms. According to ANSORP, the rate of MRSA in Vietnam is >74% of all the hospital-acquired infections with *S.aureus*; the MRSA rate for community-acquired *S.aureus* infection is approximately 30% of cases (Kang and Song, 2013). The rates of ESBL-producing *E.coli* and *Klebsiella pneumoniae* were >50% of all urinary tract infections, which is higher than the 8 participating countries in Asia. Lastly, the rate of carbapenem-resistant *Enterobacteriaceae* in Vietnam was around 3%, which was only lower than in Indonesia (5.8%) (Xu et al., 2015).

1.11 Previous studies of BSI in Vietnam

There are no routinely available national data regarding BSI in Vietnam. However, some hospitals in Vietnam have BSI researches in their own institution. A tertiary paediatric hospital had laboratory-confirmed BSI rate of 6.7% (385/5,763 cases) in neonates within one year. Gram-negative bacilli were the predominant BSI pathogen and associated with high mortality, in comparison with Gram positive pathogens ($p<0.01$)(Kruse et al., 2013). In another tertiary hospital for adults, nosocomial BSI were investigated in a prospective cohort study in 2013-2015. This study found that underlying diseases, such as diabetes, chronic kidney disease, and chronic heart diseases were associated with 30-day mortality. Central lines were identified as the most important source of nosocomial BSI in this hospital; *Candida* was the most common pathogen, followed by bacteria such as *Enterococcus*, and *Acinetobacter*(Dat et al., 2017).

There has been only one longitudinal retrospective study of BSI in Hospital for Tropical Diseases (HTD), a tertiary hospital for infectious diseases in HCMC in the south of Vietnam. The median positive rate of blood culture was 11.6% during the study period. *S. Typhi* was the predominant pathogen of BSI in the first 8 years of the study; *E.coli* and *Klebsiella* accounted for a very small portion of infections. After 2002, the rate of *S. Typhi* declined 30% annually ($p<0.01$) while there were a noticeable increased in NTS, *Cryptococcus neoformans*, and *T. marneffei* due an increasing prevalence of HIV infected patients. *S. Typhi* had a high MDR rate (92%) at the beginning of the study (1995), but then decreased gradually 18% every year, and remained at 17.6% at the end of the study (2008). In contrast, *E.coli* became increasing resistant to common antimicrobials such as aminoglycosides, fluoroquinolones, and 3rd cephalosporins(Nga et al., 2012).

1.12 What do we know and do not know about BSI in Vietnam?

Although routine surveillance data for the antimicrobial resistance in Vietnam is limited, available data outlines a picture of high rate of MDR pathogens. However, there are no current longitudinal data regarding the pathogen causing BSI, especially in some particular populations such as those infected with HIV, and those with hospital-acquired or community -acquired infections. Data contributing to understanding common antimicrobial resistance mechanisms or phenotype, including ESBLs, AmpC, KPC, and MRSA in BSI pathogens in Vietnam are also scarce. As a clinical microbiologist I think these data are valuable for management of BSI in my hospital and in Vietnam.

In previous data regarding BSI from 1994-2008 in Vietnam showed there was a shift in epidemiology from *S. Typhi* to other bacteria, including NTS and fungi, when HIV-infected patients became more commonly admitted to the hospital(Nga et al., 2012). We observed a smaller but comparative change to the HIV epidemic observed in parts of Africa. Details of invasive NTS disease in sub-Saharan Africa have been well investigated. The main epidemic invasive NTS is *Salmonella* Typhimurium ST313, which is characterized by an MDR phenotype (Feasey et al., 2015, 2012). The situation for BSI and invasive NTS disease in Vietnam is different from Africa. Malaria became uncommon in Vietnam, (Thanh et al., 2015) and anti-retroviral therapy is readily available(Kato et al., 2014). NTS BSI is present in Vietnam but information about the disease, such as clinical presentation, pathogen characteristic, and disease outcome has not been described.

In recent years, automated antimicrobial susceptibility testing using machines has become commonly adopted in Vietnamese microbiology laboratories. Comparison of automatic susceptibility testing and other manual methods have showed an acceptable performance of the automatic method for common pathogenic bacteria such as *E.coli*

and *Staphylococcus aureus* (Lee et al., 2013; Stone et al., 2007). The CLSI has recently changed the breakpoints for some antimicrobials for *Salmonella*, this reclassification has resulted in more organisms being reported as resistant to fluoroquinolone (Clinical and Laboratory Standards Institute, 2015, 2013). However, we do not know whether automatic susceptibility testing is appropriate for the interpretation of antimicrobial susceptibility data for NTS using the CLSI guidelines.

1.13 Focus, aims, and the structure of my thesis research

For a better understanding of BSI in Vietnam in an era when antimicrobial resistance is a global threat, there is an epidemic of HIV in Southeast Asia, and automated microbiological techniques are becoming more widely used, I proposed the following research questions:

Question 1.

What are the characteristics of main pathogens causing BSI and what is the trend of antimicrobial susceptibility at HTD (the tertiary site for infectious diseases in the south of Vietnam) in recent years?

In HTD, the primary antimicrobial regime for BSI is ceftriaxone as monotherapy or ceftriaxone in combination with an aminoglycoside or a fluoroquinolone. However, this antimicrobial regime is less effective if the pathogen is resistant to 3rd generation cephalosporins. We know that Vietnam is a key location for antimicrobial resistant bacteria in Asia. Therefore, it is essential to understand the common pathogens causing BSI and their antimicrobial resistance pattern in Vietnam. I aimed to retrospectively investigate all pathogens data from BSI and their corresponding clinical information over a 5 years period at HTD. I aimed to investigate the major causes of bacteraemia in this healthcare facility, their distribution by ward, their association with HIV, their

antimicrobial susceptibility patterns, and their antimicrobial resistance gene content. I also aimed to generate data regarding common resistant mechanisms such as ESBLs, AmpC lactamase, KPC, and MRSA. Based on the admission time and the blood culture collection time, data will be stratified into hospital-acquired and community acquired infections. These data will contribute to more appropriate antimicrobial regimes for BSI as well as a future antimicrobial stewardship program in this setting.

Question 2.

What are the characteristics of ESBL and AmpC lactamases, the two main types of β -lactamase in Enterobacteriaceae associated with BSIs at HTD?

β -lactamase producing Enterobacteriaceae have become increasingly common in human infections, especially in BSI. The commonest group of β -lactamases is the ESBL, which are generally plasmid-mediated. Recently, another group of β -lactamase, AmpC, has also been reported in the Enterobacteriaceae. These β -lactamases are able to hydrolyse the 3rd generation cephalosporins, which leads to failure of ceftriaxone and other 3rd generation cephalosporin antimicrobials. In HTD, ceftriaxone is the primary treatment for BSI. However, ceftriaxone is ineffective for ESBL and/or AmpC expressing bacteria. Therefore, it is essential that ESBL or AmpC activity and genes can be identified in a clinical microbiology laboratory. At HTD, manual detection method for ESBL activity has been performed since 1994; AmpC activity since 2011. However, the sensitivity and specificity of these manual methods remains unclear. I aimed to evaluate conventional methods to detect ESBL and AmpC activity in this setting in comparison with molecular methods. Multiplex PCR for common ESBL and AmpC genes will be performed for each organism with evidence of ESBL and AmpC activity. Based on the molecular screening results, the common ESBL and AmpC genes associated with BSI in this setting will be identified.

Question 3.

What are clinical and laboratory characteristics of NTS BSI in Vietnam?

It is apparent that there is a changing epidemiology of *Salmonella* infections in Vietnam and in other parts of Southeast Asia. However, the nature, the scale, and the implications of this changing epidemiology are, as yet, unknown. I think, from limited available data, that the number of invasive NTS infections in Vietnam is small in comparison to those observed in sub-Saharan Africa. I am unsure of the clinical manifestations, the potential success of the treatment regimes, and the affected patient population. I hypothesise that the epidemiology of invasive NTS infections is ecologically different to that of sub-Saharan Africa, and I predict that as hospital care, antiretroviral drugs, and nutrition is good in Vietnam that mortality is low and limited to few specific risk factors. To address this hypothesis I aimed to perform a retrospective analysis on all patients hospitalised at HTD in HCMC over a four-year period. Data will be collected from hospital records, entered into a custom designed database, stratified by HIV status, age, sex, nutrition, treatment, hospital stay, complications, gastrointestinal complaints, and outcome. Data will be described and then analysed to assess risk factors or outcome, prolonged hospital stay, complications, co-morbidities, and death. This will be the first study of its type originating from outside sub-Saharan Africa.

Question 4.

Does automatic antimicrobial susceptibility testing using the VITEK system produce antimicrobial results as reliable as conventional methods for *Salmonella*?

Since automatic antimicrobial susceptibility testing systems have become available they have been widely adopted by clinical microbiology laboratories. With advantages such as rapidity and reproducibility, the VITEK system, which is one of the most common automated system, has been evaluated with good performance for many common pathogens such as *E.coli*, *Klebsiella*, *Pseudomonas*, and *Staphylococcus aureus* (Lee et al., 2013; Stone et al., 2007; Tan and Ng, 2007). The clinical microbiology laboratory at the HTD installed a VITEK 2 compact system in 2012. This machine was routinely used for testing the antimicrobial susceptibility profiles of resistant bacteria isolated from patients within the hospital. To date, there have been no studies using the VITEK system for testing *Salmonella* antimicrobial susceptibility, with most studies using disk-diffusion or E-tests (Chiou et al., 2014; Lee et al., 2009; Lunguya et al., 2013). Therefore, I think it is necessary to understand the accuracy of VITEK susceptibility testing method for *Salmonella* by comparing VITEK method with E-tests, and the disk diffusion method. This information will be valuable for clinical microbiology laboratories in selecting appropriate antimicrobial susceptibility methods for *Salmonella* in an era of automation.

Chapter 2

Material and methods

2.1 Setting

2.1.1 Vietnam

Vietnam is a country in located in Southeast Asia; it has a landmass of 332 thousand km² and a population approaching 90 million people. According to the Ministry of Health, the infrastructure for healthcare services in Vietnam is below the national requirement. There are 23.5 hospital beds/10,000 people, which are not evenly distributed between regions and provinces. Since 1998, the Centre for Disease Control had supported Vietnam for strengthening healthcare facilities, which has included the management of HIV and other infectious diseases(Global Health, 2016). In recent years, specific infectious diseases and antimicrobial resistance have become a large burden in Vietnam. The country is now trying to tackle the spread of high level of antimicrobial resistance; this requires more attention from the healthcare systems and the government.

2.1.2 Hospital for Tropical Diseases (HTD)

HTD in HCMC is a major referral hospital for infectious diseases in the south of Vietnam. The hospital has 550 beds and receives more than 2,500 outpatients daily. More than 70% patients are citizens of HCMC; the remaining patients are from the nearby provinces. The hospital receives both adults and children and also provides healthcare service for HIV- infected patients. The HTD has 14 clinical wards including three ICUs (adult ICU, pediatric ICU, and an ICU for central nervous system infections), six adult non-ICU wards, four non-ICU pediatric wards and one HIV ward.

2.1.3 The Oxford Clinical Research Unit in Vietnam (OUCRU-VN)

Being part of the Wellcome Trust Major Overseases Programme, OUCRU-VN is a large institution for research and training in Vietnam. The headquarters of OUCRU-VN has been located within the grounds of the HTD hospital, HCMC since 1991. This alliance forms an advantage for collaboration with this tertiary referral hospital for infectious diseases and other large hospitals in HCMC. OUCRU-VN had achieved broad success in many important research areas including on central-nervous system infections, opportunistic diseases in HIV infections, enteric infections, and antimicrobial resistance.

2.2 Methods

2.2.1 Methods for chapter 3: A retrospective study of bloodstream infection in HTD from 2010-2014:

2.2.1.1 Study design and setting

This was a retrospective, descriptive study conducted on data and organisms collected over a five-year period from patients with bacteraemia at HTD, and performed in collaboration with OUCRU-VN. This study was a part of a larger study coded 15EN (HTD research code CS/ND/14/20) which was been approved by the Ethical Review Board of HTD.

2.2.1.2 Inclusion and exclusion criteria

The inclusion criteria for this analysis were every patient at HTD with a positive culture from a blood sample taken between the 1st January 2010 and 31st December 2014. There were no exclusion criteria.

2.2.1.3 Data collection

The data for this analysis was all available routine microbiology laboratory data regarding bacteraemia at HTD from 2010-2014 inclusive that was stored on the hospital computerised database. These data included; all cause bacteraemia from 2010-2014, aetiology, date of isolation, ward, sex, HIV status, antimicrobial susceptibility patterns from all bacterial isolates. Clinical data from BSI patients were not collected routinely, except for the outcome, which was derived from the data network of the hospital.

2.2.1.4 Blood culture and organism identification

Blood cultures at HTD are performed for patients in whom an infection was suspected on the basis of a fever ($>38^{\circ}\text{C}$) or who has evidence of sepsis on the basis of the presence of two or more of the following features: fever ($>38^{\circ}\text{C}$) or low temperature ($<36^{\circ}\text{C}$); tachycardia (exact level according to age); tachypnea (exact level according to age); an elevated white cell count ($>12,000\text{ cells/mm}^3$) or depressed white cell count ($<4,000\text{ cells/mm}^3$). There was no systematic change in the application of these criteria during the time course of the study. All data originating from consecutive patients admitted to the hospital who had a blood culture performed for suspected bloodstream infection between 1st January 2010 and 31st December 2014 were included in this retrospective study. Routinely, a member of the hospital staff recorded the date of blood draw, the patient's age, sex, and suspected diagnosis, the number of blood culture bottles inoculated, the result of the culture (whether positive or negative) and the susceptibility of the isolate to commonly used antimicrobial agents. These are the source data for this study.

Single venous blood culture of 8-15 mL from adults and 2-5 mL of venous blood from infants and children were routinely obtained and inoculated into BACTEC^{plus} aerobic bottles (Becton Dickenson, USA). Inoculated BACTEC bottles were incubated at 37°C in a BACTEC 9240 automated analyser for up to five days and sub-cultured when the machine indicated a positive signal. All sub-cultures were plated onto fresh sheep blood agar, MacConkey agar, and chocolate agar for bacterial isolation. Sabouraud agar plate was used only when yeast or fungi was seen on the direct smear. Plates were incubated at 37°C in air for five days and organisms were subsequently identified by standard methods including API20E and API20NE identification kits (Bio-Mérieux, France). *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

2.2.1.5 Antimicrobial susceptibility testing and interpretation

The susceptibility to relevant antimicrobial agents was determined by the modified Bauer-Kirby disc diffusion method. The antimicrobial susceptibility profiles of Gram-negative pathogens were generated using a 3rd generation cephalosporin, a fluoroquinolone, and a carbapenem. Data is presented in the number or the proportion of non-susceptible (intermediate and resistant) bacteria to a specific antimicrobial group. Non-susceptible to 3rd cephalosporins were determined using ceftriaxone for the Enterobacteriaceae (*E.coli*, *Klebsiella* spp., *S. Typhi*, and NTS), and ceftazidime for *Acinetobacter* spp. and *Pseudomonas* spp. Non-susceptible against carbapenems was determined using ertapenem and/or imipenem and/or meropenem for *E.coli*, *Klebsiella* spp., *S. Typhi*, NTS, imipenem and/or meropenem against *Acinetobacter* spp. and *Pseudomonas* spp.. If an isolate was non-susceptible to one carbapenem but susceptible to another it was considered as “non-susceptible”. Lastly, ofloxacin, ciprofloxacin or levofloxacin was used to determine the non-susceptibility against fluoroquinolones.

Detection for MRSA was performed routinely for all *Staphylococcus aureus* using cefoxitin disk screening test (MRSA was concluded if the cefoxitin zone of inhibition was <21 mm). The breakpoint zone sizes were interpreted according to CLSI guidelines.

The double disk diffusion method was used to identify ESBL activity. This method was performed using a combination of cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), Amoxicillin (20 µg)/clavulanate (10 µg). ESBL producers were identified by reduced zone sizes to third-generation cephalosporins (ceftazidime and ceftriaxone), and expansion of these zones in the presence of an inhibitor (clavulanate). Confirmatory tests for ESBL producers were performed using oxyamino-cephalosporins/β-lactam inhibitor combinations, namely cefotaxime (30 µg)/clavulanate (10 µg) and ceftazidime (30 µg)/clavulanate (10 µg). Zone sizes were measured and interpreted according to the CLSI guidelines.

2.2.1.6 Definitions

For the purposes of these analyses positive blood samples taken for culture >48 hours after admission to HTD were classified as a Hospital Acquired Infections (HAI) and samples taken within 48 hours of admission to HTD were classed as a Community Acquired Infections (CAI).

Contamination was determined in the microbiology laboratory if the organisms grown in blood culture were *Corynebacterium* spp., *Bacillus* spp., *Diphtheroid* spp., *Micrococcus* spp., and *Propionibacterium* spp. Coagulase negative *Staphylococcus* and *Burkholderia cepacia* were considered as contaminants on a case by case basis, after the agreement of the microbiology laboratory and attending clinician.

2.2.1.7 Sample size

As this was retrospective descriptive study, the sample size was dependent on the number of patients with available data according to the inclusion criteria during from 2010-2014.

2.2.1.8 Statistic method

The resulting data is presented in the form of tables and bar charts for descriptive variables i.e. the number of specific organisms per year and number of resistant organisms per year. Time trends (over the 5-year period), including the proportion of cultured isolates by year, the antimicrobial susceptibility patterns were determined by logistic regression, and odds ratios will be presented in units of time (per year). All statistical analysis were performed using R (version 3.2.3); p values of ≤ 0.05 will be considered significant.

2.2.2 Methods for chapter 4: The phenotypic and genotypic characteristics of ESBL and AmpC producing organisms associated with bacteraemia from 2011-2013:

2.2.2.1 Study design and setting

This was a retrospective study on the ESBL and AmpC producing Gram negative bacilli isolated from positive blood cultures in HTD from 2011 to 2013.

2.2.2.2 Ethics statement

Ethical approval for this study was provided by the ethical review board of HTD in HCMC.

2.2.2.3 Sample collection

HTD microbiology laboratory stored the organisms and their data for this study under a routine storage procedure. All the organisms isolated from blood cultures were fully identified and antimicrobial susceptibility tested. Identification, the antibiogram and the patients' data were saved in Labconn, computer software and manual logbooks in Microbiology laboratory. Pure organisms were stored in Brain-heart infusion glycerol and stored at -40°C. Based on the stored microbiology data, information regarding ESBL or AmpC lactamase producing Gram-negative bacilli was extracted from the Labconn. The desired stored strains were selected from the freezer and cultured on nutrient agar plate. Weak growing strains were subcultured again on nutrient agar or MacConkey agar for the optimal growth. After recovery all the strains were identified again by using the set of conventional biochemical tests (including oxidase, glucose, lactose, indole, citrate, urea, methyl red, motility test). If identification was unable to be determined by biochemical testing an API test strips (20NE and 20E) was performed. *Acinetobacter* spp., *Pseudomonas* spp. and other non-Enterobacteriaceae were excluded from this study.

2.2.2.4 Sample size

All Gram-negative bacilli bacteria that were subcultured from the frozen stock.

2.2.2.5 Antimicrobial susceptibility testing

The susceptibility to relevant antimicrobial agents was determined by the modified Bauer-Kirby disc diffusion method. Enterobacteriaceae were tested with discs

containing amikacin(10 µg) , ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), imipenem (10 µg), ertapenem (10 µg), meropenem (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg), amoxicillin/ acid clavulanic (amoxicillin 20 µg / acid clavulanic 10 µg), trimethoprim/ sulfamethoxazole (trimethoprim 1.25 µg / sulfamethoxazole 23.75 µg), ticarcillin/ acid clavulanic (ticarcillin 75 µg/ acid clavulanic 10 µg) , piperacilline/ tazobactam (piperacillin 100 µg / tazobactam 10 µg) and cefoxitin (10 µg). The breakpoint zone sizes were interpreted according to CLSI guidelines. As before the double disk diffusion method was used to identify ESBL activity.

Phenotypic AmpC activity was detected using two different tests. First, I measured zone sizes using a combination of cefoxitin and an alternative third- and fourth-generation cephalosporin, where an AmpC positive organism would be resistant to cefoxitin and exhibit reduced susceptibility to the alternative third generation cephalosporin (ceftriaxone 30 µg) and complete susceptibility to the fourth generation cephalosporin (cefepime). Secondly, I determined inducible AmpC phenotypes by assessing reduced zone sizes against a third generation cephalosporin (ceftriaxone 30 µg or ceftazidime 30 µg) in the presence of imipenem (30 µg) as an inducing substrate (Figure 2.1). AmpC producing organism was phenotypically determined by cefoxitin disk screening test and AmpC induction test (Table 2.1).

Phenotypic screening for ESBL was performed by both double disc method and combination disc method. Interpretation was described in Table 2.1 and illustrated on Figure 2.1.

Figure 2. 1 Representative results of the double disk diffusion test

(A) for ESBL production and the AmpC disk test (B). Abbreviations. AMC: amoxicillin, CTX: cefotaxime, CAZ: ceftazidime, CRO: ceftriaxone, CLA: clavulanate, FEP: cefepime, FOX: ceftiofur, IPM: imipenem

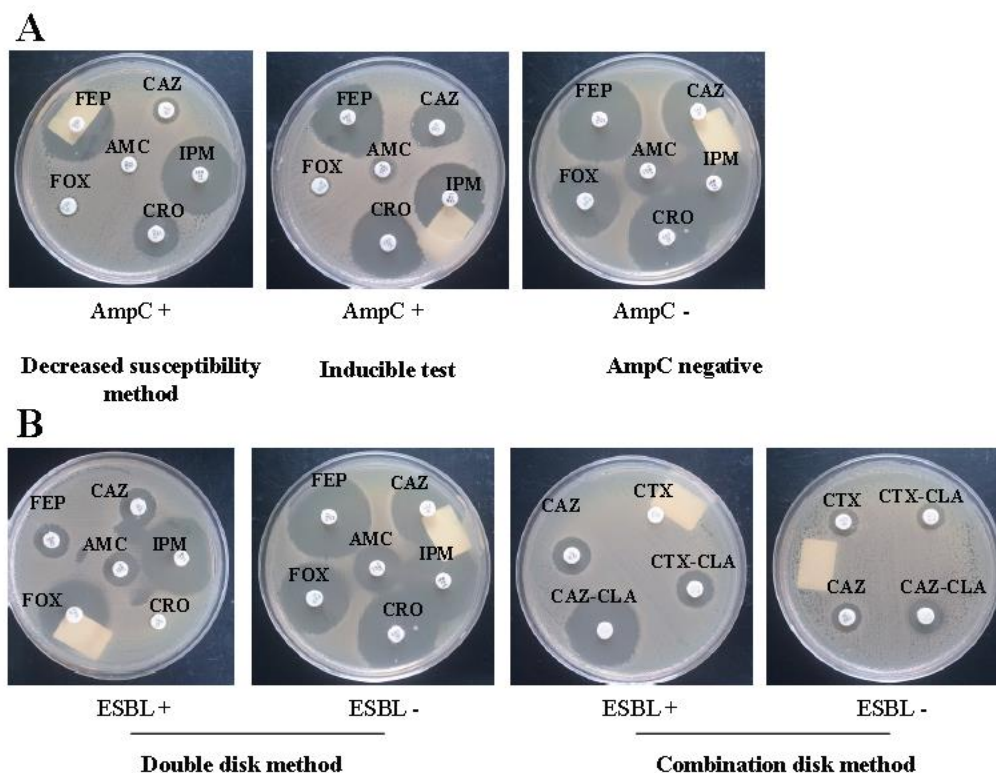


Table 2. 1 Interpretation of ESBL and AmpC lactamase from detection methods

Mechanism	Method	Positive result
ESBL	Combination disc test	Special “champagne-cork” or “key-hole” images between amoxicillin/clavulanate and ceftazidime/ceftriaxone.
	Double-disc test	Inhibition zone diameter of either (cefotaxime-clavulanate) – cefotaxime $\geq 5\text{mm}$ (ceftazidime-clavulanate) – ceftazidim $\geq 5\text{mm}$
AmpC lactamase	Cefoxitin disk screening test	Inhibition zone diameter of : Ceftriaxone $< 23\text{ mm}$ and/or ceftazidime $< 21\text{mm}$ <i>plus</i> Cefepime $\geq 24\text{ mm}$ <i>plus</i> Cefoxitin $< 17\text{mm}$
	AmpC induction test	A flattening zone of ceftazidime/ceftriaxone toward imipenem

2.2.2.6 Genotypic screening of ESBL and AmpC genes

Blood isolates collected from the same patient at two different time-points with the same phenotypic AMR profile were denoted as duplicates and only one was subsequently selected for further PCR analysis. If the AMR profile of the two isolates selected differed, both were selected for PCR analysis. Multiplex PCR reactions were used to detect ESBL (*bla*_{CTX-M} subtypes) and AmpC genes. Other β -lactamase genes, *bla*_{TEM}, *bla*_{OXA}, and *bla*_{SHV} were also detected by multiplex PCR using the following cycling conditions, initial denaturation step at 95°C for 15 minutes, followed by 25 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing at 57°C for 40 seconds, and primer extension at 72°C for 30 seconds. After the last cycle, a final extension step at 72°C for 10 minutes was added. PCR amplicons were examined by agarose gel (BioRad) electrophoresis made up to a concentration of 1.5% (w/v). The primers used in this study are shown in Table 2.2.

All positive ESBL (*bla*_{CTX-M}) and AmpC (*bla*_{CTT}) PCR amplicons were sequenced to further subtype these genes. DNA was extracted using the Agencourt AMPure XP PCR purification system (Beckman Coulter) and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied biosystems) on the 3130 genetic analyser (Applied biosystems). DNA sequences were compared against the National Centre of Biotechnology Information (NCBI) GenBank sequence database using the BLAST algorithm and gene variants were subsequently deduced based on sequence similarity.

Table 2. 2 Primers for the multiplex PCR assays to detect ESBL and AmpC targeted genes

Multiplex	Resistance mechanism	Target gene	Name	Sequence (5'-3')	Expected amplicon size (bp)	Reference
I	AmpC	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXF ^a	GCTGCCAAGGAGCACAGGAT	520	Pérez-Pérez J. et al (Pérez-Pérez and Hanson, 2002)
I	AmpC	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	MOXR ^a CITF	CACATTGACATAGGTGTGGTGC TGGCCAGAAGTACAGGCCAAA	462	Pérez-Pérez J. et al (Pérez-Pérez and Hanson, 2002)
I	AmpC	DHA-1, DHA-2	CITR DHAF	TTTCTCCTGAACGTGGCTGGC AACTTTCACAGGTGTGCTGGGT	405	Pérez-Pérez J. et al (Pérez-Pérez and Hanson, 2002)
I	AmpC	ACC	DHAR ACCF	CCGTACGCATACTGGCTTTGC AACAGCCTCAGCAGCCGGTTA	346	Pérez-Pérez J. et al (Pérez-Pérez and Hanson, 2002)
I	AmpC	MIR-1T ACT-1	ACCR EBCF	TTCCGCCGAATCATCCCTAGC TCGGTAAAGCCGATGTTGCGG	302	Pérez-Pérez J. et al (Pérez-Pérez and Hanson, 2002)
I	AmpC	FOX-1 to FOX-5b	EBCR FOXF	CTTCCACTGCGGCTGCCAGTT AACATGGGGTATCAGGGAGATG	190	Pérez-Pérez J. et al (Pérez-Pérez and Hanson, 2002)
II	ESBL	CTX-M1	FOX M1F	CAAAGCGCGTAACCGGATTGG AAAAATCACTGCGCCAGTTC	415	Woodford N. et al (Woodford et al., 2004)
II	ESBL	CTX-M2	M1R M2F	AGCTTATTCATCGCCACGTT CGACGCTACCCCTGCTATT	552	Woodford N. et al (Woodford et al., 2004)
II	ESBL	CTX-M9	M2R M9F	CCAGCGTCAGATTTTTCAGG CAAAGAGAGTGCAACGGATG	205	Woodford N. et al (Woodford et al., 2004)
II	ESBL	CTX-M8/25	M9R M8F	ATTGGAAAGCGTTCATCACC CGCGTTAAGCGGATGATGC	666	Woodford N. et al (Woodford et al., 2004)
III	ESBL		M25F	GCACGATGACATTCGGG	327	Nguyen et al (Thi
			M8/25R TSO-T-F	AACCCACGATGTGGGTAGC TGCGGTATTATCCCGTGTTG	296	

III	ESBL	TEM variants including TEM-1 & TEM-2 SHV variants including SHV-1	TSO-T-R	TCGTCGTTTGGTATGGCTTC	713	Khanh Nhu et al., 2010)
			TSO-S-F	AGCCGCTTGAGCAAATTAAAC		
III	ESBL	OXA-1, OXA-4 & OXA-30	TSO-S-R	ATCCCGCAGATAAATCACCAC	564	Nguyen et al (Thi Khanh Nhu et al., 2010)
			TSO-O-F	GGCACCAGATTCAACTTTCAAG		
			TSO-O-R	GACCCCAAGTTTCCTGTAAGTG		

2.2.2.7 Statistical analysis

Statistical analysis was performed in R. Comparisons between HAI and CAI antimicrobial resistance instance rates was assessed using the Fisher's exact test (two-tailed). A p -value <0.05 was considered statistically significant.

2.2.3 Methods for chapter 5: A retrospective study of BSI caused by nontyphoidal *Salmonella* in HTD from 2008-2013:

2.2.3.1 Study design

The study population consisted of all individuals from which an NTS organism was isolated alone or in combination with an additional pathogen in blood culture from January 2008 through June 2013. This source data was collected from routine microbiology laboratory logbooks in which data from positive and negative blood culture are recorded. Patients with multiple positive blood cultures for the same NTS serogroup and antimicrobial susceptibility profile were considered to be a single case.

2.2.3.2 Ethical approval

The protocol for this study was submitted to the Ethical and Scientific Committee of HTD in 2012 and got approval at the same year (HTD study code: CS/ND/12/26). The OUCRU application code was 07EN.

2.2.3.3 Data collection and definition of disease outcome

A patient record form was used to collect clinical and laboratory data from the hospital chart for every patient. Clinical data recorded on admission included sex, HIV status (HIV diagnosed according to the WHO guidelines), axillary temperature, presence of co-infection and hospital outcome. Outcome was classified based on clinician notes as follows: (1) recovery or improvement, (2) worsening status on discharge (often deteriorating patients taken from hospital by their relatives to die at home – a common custom in Vietnam), (3) death or (4) transfer to a different hospital (patient's condition

was unchanged but transferred to other hospital for specific treatment or surgery intervention, or patient left against medical advice). Outcome 2 and outcome 3 were considered to be fatal. Laboratory data was comprised of standard hematology and biochemical testing from hospital records on the day of admission.

2.2.3.4 Statistical analysis of clinical and laboratory data

Clinical and laboratory data were compared between groups using Fisher's exact or Kruskal-Wallis tests for categorical and continuous data, respectively. We performed univariable and multivariable logistic regression to evaluate covariates that were independently associated with fatal outcome. Covariates selected for multivariable analysis *a-priori* included age, sex and immunosuppression (HIV status, chronic hepatitis), in addition to other fixed demographic or clinical covariates that were significantly associated ($p < 0.05$) with outcome from the univariate analysis. All statistical analyses were performed using Stata version 11 (StataCorp, College Station, TX, USA). Plots were made in R (v3.1.1; R Foundation for Statistical Computing).

2.2.3.5 Microbiological procedures

Organisms were identified by standard methods including API20E identification kits (Bio-Mérieux, Craponne, France). Specific grouping antisera were used to identify the serogroup of the isolated *Salmonella* on original culture. Vi antisera (along with 0:9) was used to identify *S. Typhi*; these were excluded from all analyses. All NTS isolated from blood cultures were stored in Brain Heart Infusion (BHI) glycerol at -70°C . For the purposes of this study all NTS isolated were recovered on MacConkey agar and subjected to re-identification and antimicrobial susceptibility testing. Re-identification of *Salmonella* serogroups was performed using specific grouping antisera as before.

Antimicrobial susceptibility testing was performed on Muller-Hinton agar against ampicillin, amoxicillin/clavulanate, azithromycin, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, ofloxacin and trimethoprim-sulfamethoxazole, using the disk diffusion method as recommended by CLSI guidelines.

To further characterize the iNTS isolates, all organisms were genotyped (and molecular serotyped) using multi-locus sequence typing (MLST) following previously described methods. Briefly, a set of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA* and *thrA* – primer sequences accessed at <http://mlst.warwick.ac.uk/mlst/dbs/Senterica>) (Table 2.3) were PCR amplified using template DNA extracted from each isolate after boiling bacterial colonies in phosphate buffered saline. PCR amplicons were cleaned using Agentcourt Ampure XP (Beckman Coulter) and were sequenced in both directions using BigDye Terminator v3 (Applied Biosystems, USA) followed by capillary sequencing on a 3130XL Genetic Analyzer (Applied Biosystems, USA). All sequences were manually trimmed to align to a reference sequence and were submitted to the previously mentioned MLST database for allelic profile and molecular serotyping (i.e. inferring serovar from MLST profile). A minimum spanning tree was created using the allelic profiles (variation in number of alleles between isolates of the seven housekeeping genes) using Bionumerics software (Applied Mathematics).

Table 2. 3 Primers for primary PCR to detect 7 house-keeping genes of nontyphoidal *Salmonella*(Noda et al., 2011)

House-keeping gene	Sequence (5'-3')
<i>thrA</i>	GTC ACG GTG ATC GAT CCG GT
<i>purR</i>	ATG TCT TCC CGC AAT AAT CC
<i>sucA</i>	AGC ACC GAA GAG AAA CGC TG
<i>hisD</i>	GAA ACG TTC CAT TCC GCG CAG AC
<i>aroC</i>	CCA CAC ACG GAT CGT GGC G
<i>hemD</i>	CCA CAC ACG GAT CGT GGC G
<i>dnaN</i>	ATG AAA TTT ACC GTT GAA CGT GA

2.2.4 Method for chapter 6: A descriptive study about the progress of antimicrobial resistance of *Salmonella* species and comparison between several susceptibility testing methods for *Salmonella* species 2008-2015:

2.2.4.1 Study design and setting

This was a descriptive study testing the antimicrobial susceptibility profiles of *Salmonella* spp. isolated from blood cultures at the HTD over 8 years (2008-2015) period. All organisms were tested by 3 methods: E-test, disk diffusion, and automatic VITEK 2 system.

2.2.4.2 Ethical approval

This study was a part of the large study 15EN (HTD research code CS/ND/14/20), which was approved by the Ethical Review Board of HTD.

2.2.4.3 Sample collection

All *Salmonella* organism isolated from blood cultures were routinely stored in Microbiology laboratory of HTD. The strains were stored at -40°C in glycerol brain-heart-infusion cryotubes.

When recovered, all *Salmonella* organisms were extracted from the stock and subcultured for two generations on MacConkey agar. Young and pure colonies were picked up for re-identification and antimicrobial susceptibility testing. All *Salmonella* were re-identified by routine biochemical tests and antisera. Isolates were identified as *S. Typhi*, *S. Paratyphi* A, B, C or *Salmonella* spp. using conventional biochemical testing and *Salmonella* polyvalent antisera (polyvalent O, O2, O4, O9 and Hd).

2.2.4.4 Antimicrobial susceptibility testing

All *Salmonella* were tested by disc-diffusion, E-test and by automatic susceptibility test by VITEK system at together. The antimicrobials tested by disc-diffusion (Kirby-Bauer), and E-test were nalidixic acid, ofloxacin, ciprofloxacin, azithromycin, trimethoprim-sulfamethoxazole, ampicillin and ceftriaxone. These antimicrobials are common used for Salmonellosis treatment at HTD. Chloramphenicol was performed by disk-diffusion to determine MDR resistant *Salmonella* (resistant to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole).

For the VITEK system, there are no available antimicrobial susceptibility cards containing all the antimicrobial listed above. Nalidixic acid and azithromycin were not been produced in any commercial cards VITEK cards available in Vietnam at the time of this work. Ofloxacin and ciprofloxacin are also not combined on any card, so that I selected a VITEK card containing only one. Finally, I used the most appropriated VITEK AST card (AST GN67) which contained ampicillin, ceftriaxone, ciprofloxacin, trimethoprim-sulfamethoxazole, and variety of less clinically relevant antimicrobials.

2.2.4.5 Interpretation of antimicrobial testing result

Interpretation of all testing antibiotics is described in Table 2.4.

Susceptibility interpretation for all antimicrobials (except ofloxacin) was determined using the CLSI guidelines M100-S25 (2015)(Clinical and Laboratory Standards Institute, 2015). Susceptibility results are presented as Susceptible (S), Intermediate (I) and Resistant (R). There were no diameter breakpoints for ofloxacin in latest CLSI guidelines (CLSI deleted the paper breakpoint for this antimicrobial from 2013).

However, some research groups have evaluated the breakpoints for ofloxacin using the presence of resistant genes and MIC. According to Parry et al (2010), an interpretation

of *Salmonella* which is to susceptible against ofloxacin by disk inhibition zone diameter ≤ 28 mm (or MIC ≤ 0.25 $\mu\text{g/ml}$) has a sensitivity and specificity of $>94\%$ (Parry et al., 2010). However, the author did not suggest breakpoints for intermediate and resistance mutations were screened in the *gyrA* gene only. In 2014, a group in the US set a zone diameter breakpoint for ofloxacin: ≥ 25 mm (Susceptible), 16 to 24 mm (Intermediate), and ≤ 15 mm (Resistant) using the MIC breakpoints in CLSI M100-S23 (Sjölund-Karlsson et al., 2014). This diameter breakpoint was established from testing 100 *Salmonella* whose with resistance mechanisms identified by PCR and sequence in the both *gyrA* and *parC* genes.

Table 2. 4 Susceptibility interpretation of *Salmonella* species by Clinical Laboratory Standard Institute M100-S25 (2015) and other researches

Antimicrobial agent	Zone diameter interpretive criteria (mm)			MIC interpretive criteria (µg/ml)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Ampicillin	≥ 17	14–16	≤ 13	≤ 8	16	≥ 32
Ceftriaxone	≥ 23	20–22	≤ 19	≤ 1	2	≥ 4
Azithromycin	≥ 13	-	≤ 12	≤ 16	-	≥ 32
Ofloxacin	≥ 25(Sjölund-Karlsson et al., 2014)	16-24(Sjölund-Karlsson et al., 2014)	≤ 15 ^{2v58}	≤ 0.12	0.25-1	≥ 2
Ciprofloxacin	≥ 31	21-30	≤ 20	≤ 0.06	0.12-0.5	≥ 1
Nalidixic acid	≥ 19	14-18	≤ 13	≤ 16	-	≥ 32
Trimethoprim-sulfamethoxazol	≥ 16	11-15	≤ 10	≤ 2/38	-	≥ 4/76
Chloramphenicol	≥ 18	13-17	≤ 12	≤ 8	16	≥ 32

2.2.4.6 E -test method as a reference

Although agar dilution is considered as the best reference method for antimicrobial susceptibility testing, it is not a common method for antimicrobial testing in clinical laboratory due to procedure. The E-test method has been reported to produce an acceptable antimicrobial result as comparison for the agar dilution method (Amsler et al., 2010; Heijden et al., 2007; Tan and Ng, 2007). Therefore, this study utilised the E-test method as a reference to evaluate the Kirby-Bauer method and the VITEK system.

2.2.4.7 Definition of categorical agreement and errors

Categorical agreement between methods was defined as the percentage of isolates tests results that exhibit the same susceptibility rank with the reference method (E-test method). A **Minor error** was defined as susceptible or resistant with the comparison method (Kirby-Bauer or VITEK) and intermediate by using the reference method (E-test) and vice versa. A **Major error** was defined as the resistant with the Kirby-Bauer or VITEK methods and susceptible by E-test method. A **very major error** was defined as susceptible with the compared method (Kirby-Bauer or VITEK) and resistant by the E-test (Ling et al., 2001).

2.2.4.8 Antimicrobial resistant data

Data are presented in the form of tables and bar charts for the descriptive variables i.e. number of specific *Salmonella* species (*S. Typhi*, *S. Paratyphi*, and NTS) per year and number of resistant organisms per year. The resistance profiles of specific *Salmonella* spp. against each antimicrobial are presented by the MIC₉₀ and the MIC₅₀.

2.2.4.9 Comparison between antimicrobial testing methods

ISO 20775:2007 standard: The comparison of antimicrobial testing methods was conducted according to ISO 20776:2007. ISO 20776:2007 is an international standard for evaluation of performance of antimicrobial testing methods (International Organisation for Standardization, 2007). I intended to compare the susceptible, intermediate and resistant results of the Kirby-Bauer (disk diffusion test) and VITEK method in comparison with the E-test method (reference method). The comparison parameters included category agreement (CA), very major discrepancy (VMD), major discrepancy (MJD) and minor discrepancy (MD). Acceptable results are category agreement (CA) $\geq 90\%$, and a VMD and MD $\leq 3\%$ as recommended by the ISO 20776:2007 (International Organisation for Standardization, 2007). However, according to CLSI M23-A2, acceptable results are $<1.5\%$ for very major errors, $<3\%$ for major errors, and $<10\%$ for minor errors.

Comparison formulas

Categorical agreement (CA): agreement of antimicrobial susceptibility testing result between the comparison test and the reference test. The result is expressed as a

$$CA = \frac{NCA \times 100}{N}$$

percentage.

(NCA: the number of isolates with the same categories (SIR) with the reference method;

N: the number of all tested isolates)

Very major discrepancy (very major error) (VMD): test result by the reference method interpreted as resistant and a comparative test result of susceptible. The result is expressed as a percentage.

$$\text{VMD} = \frac{\text{NVMD} \times 100}{\text{NRREF}}$$

(NVMD: the number of tests that result in a VMD; NRREF: the number of resistant bacterial isolates as determined by the reference method)

Major discrepancy (Major error) (MJD): Test results are resistance by the comparison test and susceptible by reference method. The result is expressed as a percentage.

$$\text{MJD} = \frac{\text{NMD} \times 100}{\text{NSREF}}$$

(NMD: the number of tests that results in a major discrepancy (MD); NSREF: the number of susceptible isolates as determined by the reference method)

Minor discrepancy (MnD) (minor error): Test result by the reference method interpreted as resistant or susceptible and a comparison test result of intermediate; or a reference result interpreted as intermediate and a compared test result of resistant or susceptible. The result is expressed as a percentage.

$$\text{MnD} = \frac{\text{NMD} \times 100}{\text{N}}$$

(NMD is the number of tests that result in an MD; N is the total number of isolates tested)

Chapter 3

Bloodstream infections at the Hospital for Tropical Diseases in Ho Chi Minh City from 2010 to 2014

3.1 Aims of Chapter

The main objectives of this study were to identify and describe the major bacterial pathogens associated with BSI at HTD over a five-year period. Additionally, I sought to investigate trends in antimicrobial resistant profiles of the main pathogens, and to determine the source of infection (community or hospital) of isolates to identify the principal location associated with the acquisition of acquiring a bacterial infection associated with a multi-drug resistance.

3.2 Introduction

In HTD, BSI is an important disease that requires close management and intensive treatment. Every year, approximately 7,000-8,000 blood cultures are performed in this hospital. In local general practice a blood culture is requested for every suspected case of BSI alongside other general blood tests. Systematically, all HIV-infected cases have blood culture after admission. Identification and susceptibility result from blood culture play an important role in antimicrobial selection and therapy amendment in individual cases. Antibigrams at HTD are required to be reported annually but not for specific for BSI pathogens. Important antimicrobial resistance phenotypes such as ESBL, AmpC lactamases, MRSA, carbapenemase-resistance, vancomycin-resistant enterococcus, have been found in this hospital in recent years but their circulating and commonality have not been determined. This data chapter develops an understanding of BSI at HTD over a five-year period. Specially, I describe the characteristics of blood cultures,

including positive rate, contamination rate, and outcome. I additionally determine pathogenic organisms causing BSI in HTD in the context of proportion, initial infection source, ICU infection, and outcome categories. Lastly, I assessed the trend of the antimicrobial susceptibility patterns of common pathogens versus selected antimicrobial groups.

3.3 Results

3.3.1 Overall characteristics of blood culture in HTD

Over the five years (2010-2014) of available data from HTD there were 47,304 blood samples submitted to the microbiology laboratory for culture (Table 3.1). The number of blood samples taken for culture were comparable in 2010 and 2011 (<9,000) but then increased to more than >9,000 (>10,000 in 2013) in the following years. Over this five-year period, 3,852 samples exhibited growth, resulting in an overall prevalence of 8.14% (3,852/47,304) positive blood cultures. From the 3,852 (8.1%) positive samples, 3,287 (6.9% of 47,304) were considered to non-contaminant and 565 (1.2% of 47,304) were considered to be contaminant. Therefore, the median rate of non-contaminant positive blood cultures was 7.0% (range; 6.1-7.3); the lowest rate of positive blood cultures was in 2011 (541/8,781; 6.1%) and the highest rate was in 2012 (715/9,774; 7.3%). A microbiology consultant and a clinician attending the affected patient made the final decision on every potentially contaminated blood culture. The most common contaminants in blood culture were *Burkholderia cepacia* (329/565; 58.2%) and coagulase-negative *Staphylococcus* (206/565; 36.5%). Notably, over two thirds (384/565; 68%) of the contaminated blood cultures were taken from patients on the HTD HIV ward.

3.3.2 Bloodstream infections by ward

HTD has 14 clinical wards, which include three ICUs wards (adult ICU, paediatric ICU, and central nervous system infection ICU), six adult general wards, four general paediatric wards, and one HIV ward. The prevalence of positive blood cultures in each of the 14 HTD wards over the study period is described in Table 3.2. The adult ICU and HIV ward accounted for the largest proportion of positive blood samples (23.8%; 780/1,091 and 22.3%; 762/1,091; of all positive blood samples, respectively). The HIV ward additionally had the greater proportion of positive blood samples from all the wards in 2010 and 2011. However, from 2012 the adult ICU produced a greater proportion of positive blood than other wards. A third (1,092/3,287; 33%) of all positive blood culture originated from the three ICUs.

Table 3. 1 The number and proportion of total blood cultures, positive blood cultures and contaminated blood cultures at HTD, 2010-2014

Year	No of admissions	No of blood cultures collection (% admission)	No of positive blood cultures (%admission)	No of contaminated blood cultures (%collection)	No of true positive blood cultures (%admission)
2010	45,695	8,777 (19.2)	782 (1.7)	143 (1.6)	639 (1.4)
2011	48,017	8,781 (18.3)	621 (1.3)	80 (0.9)	541 (1.1)
2012	51,151	9,774 (19.1)	869 (1.7)	154 (1.6)	715 (1.4)
2013	45,910	10,346 (22.5)	841 (1.8)	115 (1.1)	726 (1.6)
2014	44,321	9,631 (21.7)	739 (1.7)	73 (0.8)	666 (1.5)
Total	235,094	47,309 (20.1)	3,852 (1.6)	565 (1.2)	3,287 (1.4)

Table 3. 2 The number and proportion of positive blood cultures by ward at HTD, 2010-2014

Clinical ward	No of admission	No (%) of true positive cultures	% of true positive culture/admissions
ICU wards	25,933	1,091 (33.2)	4.2
Adult ICU	6,566	780 (23.8)	11.9
Paediatric ICU	13,130	87 (2.6)	0.7
Central nervous system ICU	6,237	224 (6.8)	3.6
HIV ward	12,406	962 (22.3)	7.8
Non- ICU adult ward	98,320	1,126 (34.9)	1.2
Infection ward A	15,975	311 (9.5)	1.9
Infection ward B	13,407	372 (11.3)	2.8
Infection ward C	19,873	118 (3.6)	0.6
Infection ward D	18,345	93 (2.8)	0.5
Internal ward A	17,063	70 (3.1)	0.4
Internal ward B	13,657	162 (4.9)	1.2
Children Wards	93,555	108 (3.3)	0.1
Children ward A	25,536	30 (0.9)	0.1
Children ward B	21,613	25 (0.8)	0.1
Children ward C	23,656	17 (0.5)	0.07
Children ward D	22,750	36 (1.1)	0.2
Total	217,808	3,287 (100)	1.5

In the non-ICU wards the majority of positive blood cultures originated from infection ward B, (11.3% (372/1,091) of all positive cultures) which receives patients with prolonged fever, and Infection ward A, (9.3%(311/1,091) of all positive cultures), which receives patients with severe hepatitis.

3.3.3 Outcome

The characteristics of BSI stratified by ICU admission, outcome, and sex are presented in Table 3.3. The patients' outcome was obtained from 2,572 cases, which accounted for 66.7% (2,572/3,287) of the total blood culture positive study population. Missing outcome data was due to unavailable information on the hospital database, mostly in 2014 due to storage error (missing 96.4% (642/666) outcome data). For the purposes of this work, outcome was categorized as good (discharged with recovery), bad (death or discharged to die home (a local custom, called moribund)) or unknown (patient transferred to another hospital or self-discharges). I found that 1,667/ 2,572 cases (64.8%) had a good outcome and 619/2,572 cases (24%) had a bad outcome. The overall mortality rate for those with a positive blood culture was 3.4% (87/2,572); a further 20.6% (532/2,572) were recorded as moribund. The mortality rate (bad outcome) was 24.5% (155/633) in 2010, 25.8% (133/516) in 2011, 26.3% (183/696) in 2012, and 20.5% (144/703) in 2013.

Table 3. 3 The number and proportion of positive blood cultures by aetiology of infection, outcome, and ICU hospitalization at HTD, 2010-2014.

	Total N(%)	Etiology of infection N=2,539				Outcome N=2,572				ICU admission N=2,325			
		CA	HA	OR	<i>p</i>	Good	Bad	OR	<i>p</i>	Yes	No	OR	<i>p</i>
	3,287	2,129 (83.9)	410 (16.1)			1,667 (64.8)	619 (24.1)			1,091 (46.9)	1,234 (53.1)		
Gender													
<i>Male</i>	2,102	1,374 (65.4)	275 (13.1)	0.97	0.3	1,037 (49.3)	443 (21.1)	17.3	<0.001	709 (33.7)	686 (32.6)	21.29	<0.001
Age		2,129	410			1,667	619			1,091	1,234		
<i>Age <5 years</i> (baseline)	141 ^a	76 (53.8)	29 (20.6)	1	...	99 (70.2)	2 (1.4)	1	...	56 (39.7)	85 (60.3)	1	...
<i>Age 5-15 years</i>	56 ^b	35 (62.5)	4 (7.1)	4.9	0.03	30 (53.6)	5 (8.9)	8.1	0.05	30 (53.6)	26 (46.4)	3.3	0.07
<i>Age 16-60 years</i>	2,476 ^c	1,648 (66.6)	285 (11.5)	12.7	<0.001	1,205 (48.7)	542 (21.9)	39.2	<0.001	706 (28.5)	813 (32.8)	2.7	0.1
<i>Age >60 years</i>	614 ^d	370 (60.3)	92 (15.0)	3.0	0.08	333 (54.2)	70 (11.4)	15.6	<0.001	299 (48.7)	310 (50.5)	4.39	0.04

Table shows aetiology of infection of 2,539 BSI cases, outcome at discharge of 2,572 BSI cases and of 2,325 cases stratified by ICU/non-ICU care. Pairwise comparison using X² test, $p < 0.05$ was considered as significant. CA: community-acquired infection, HA: hospital-acquired infection. ICU category was determined based on the site of the blood culture collection.

^aAge < 5 years: 36 missing in etiology, 40 missing in outcome group;^b Age 5-15 years: 17 missing in etiology, 21 missing in outcome group;

^c Age 16-60 year: 543 missing in etiology, 729 missing in outcome group, 957 missing in ICU admission group;^d Age >60 years: 152 missing in etiology group, 211 missing in outcome group, 5 missing in ICU admission group.

3.3.4 Characteristics of patients with bloodstream infections

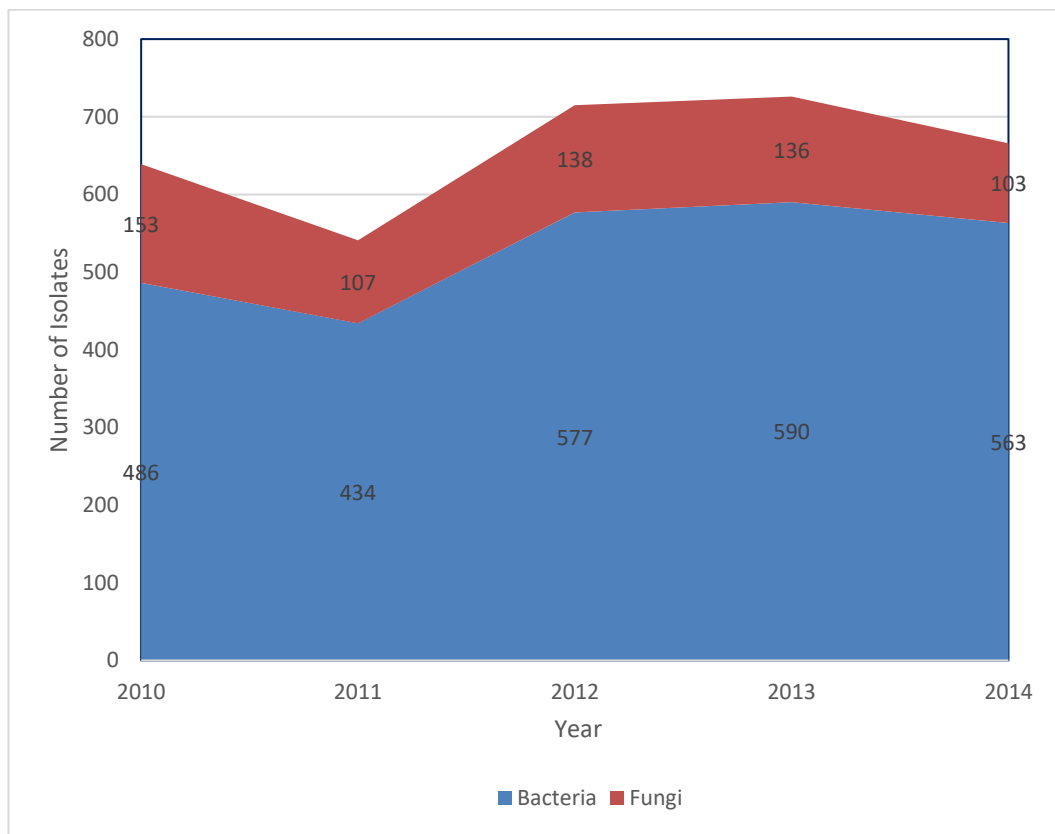
In the 3,287 clinically relevant bloodstream infection episodes, 197 (6%) cases were in children aged <15 years, 2,476 (75.3%) were within those aged 15-60 years, and 614 (18.7%) of cases were in patients aged >60 years. The mean age was of those with a clinically relevant bloodstream infection was 42 years old (IQR 29-56). In total 64% (2,102/3,287) of clinically relevant bloodstream infection episodes was in males. This distribution was more apparent in those aged under 60 years; 1,828 cases in males vs. 845 cases in females. However, there were more female than male patients (340 cases vs. 274 cases, respectively) in the elderly age group (those aged >60 years). In the 2,539 BSI with definable aetiology of infection, 16.1% (410 cases) was hospital-acquired infection. Patients aged between 5 and 15 years, 15 and 60 years and more than 60 years were more commonly associated with BSI in community ($p<0.05$) (Table 3.3).

3.3.5 Significant pathogens

Bacteria and fungi pathogens were commonly isolated in bloodstream infection at HTD from 2010 to 2014. Overall, the proportion of bacteria isolation was four times higher than fungi; 2,650 cases (80.6%) vs. 637 cases (19.4%), respectively. The relative proportion of bacteria isolated from blood increased gradually every year (2010: 76%, 2011: 80%, 2012 and 2013: 81%, 2014: 85%) (Figure 3.1).

From the 2,650 bacterial pathogens isolated over the study period, Gram-negative bacilli were the most prevalent (1,748/2,650; 65.9%), followed by Gram-positive cocci (886/2,650; 33.4%), Gram-negative cocci (8/2,650; 0.3%), and Gram-positive bacilli (8/2,650; 0.3%) (Table 3.4)

Figure 3. 1 The proportion of significant bacterial and fungal isolates in bloodstream infection at HTD from 2010 to 2014.



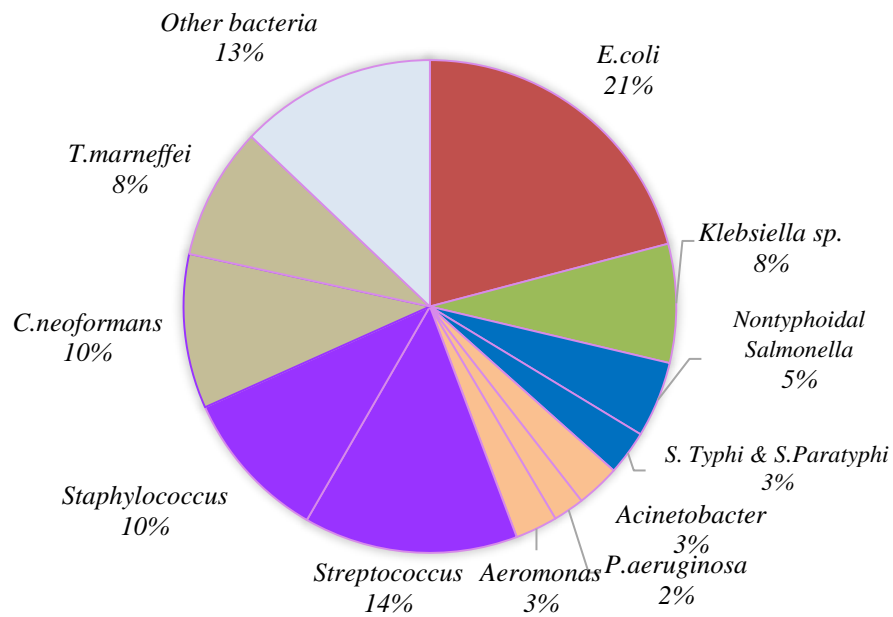
Plot showed the number of cases that had blood culture grew bacteria (blue area) or fungi (red area) in every year

Table 3. 4 The number and proportions of Gram-negative, Gram-positive, fungal pathogens isolated from bloodstream infections at HTD from 2010 to 2014

Pathogen ^a	2010	2011	2012	2013	2014	Total
<i>Gram negative bacteria</i>						
<i>Escherichia coli</i>	103 (16.12)	97 (17.92)	168 (23.50)	163 (22.45)	156 (23.42)	687 (20.9)
<i>Klebsiella spp.</i>	60 (9.38)	35 (6.46)	55 (7.69)	54 (7.44)	52 (7.81)	256 (7.9)
Non-typhoid <i>Salmonella</i>	35 (5.48)	33 (6.09)	31 (4.48)	31 (4.96)	34 (5.10)	164 (5.0)
<i>Salmonella</i> Typhi	21 (3.28)	14 (2.58)	20 (2.80)	9 (1.24)	7 (1.05)	71 (2.7)
<i>Salmonella</i> Paratyphi A	0 (0.00)	1 (0.18)	1 (0.14)	6 (0.83)	17 (2.55)	25 (0.8)
<i>Burkholderia pseudomallei</i>	13 (2.03)	13 (2.40)	15 (2.10)	19 (2.62)	14 (2.10)	74 (2.3)
<i>Burkholderia cepacia</i>	0 (0.00)	15 (2.77)	10 (1.40)	1 (0.14)	6 (0.90)	32 (1.0)
<i>Acinetobacter</i>	16 (2.50)	19 (3.51)	24 (3.36)	25 (3.44)	10 (1.50)	94 (2.9)
<i>Pseudomonas aeruginosa</i>	14 (2.19)	9 (1.66)	18 (2.52)	12 (1.65)	14 (2.10)	67 (2.0)
<i>Aeromonas</i>	11 (1.7)	15 (2.8)	14 (1.95)	26 (3.58)	25 (3.75)	91 (2.8)
<i>Gram positive bacteria</i>						
<i>Staphylococcus aureus</i>	50 (7.82)	47 (8.69)	50 (6.99)	68 (9.37)	57 (8.56)	272 (8.3)
CNS	39 (6.10)	6 (1.10)	3 (0.42)	3 (0.41)	5 (0.75)	56 (1.7)
<i>Streptococcus suis II</i>	34 (5.32)	30 (5.54)	18 (2.51)	27 (3.71)	14 (2.1)	123 (3.7)
<i>Streptococcus pneumoniae</i>	16 (2.50)	20 (3.69)	20 (2.80)	32 (4.41)	26 (3.90)	114 (3.5)
Other Streptococci	44 (6.9)	29 (5.36)	60 (8.39)	41 (5.64)	51 (7.66)	225 (6.9)
<i>Fungi</i>						
<i>Cryptococcus neoformans</i>	80 (12.51)	63 (11.64)	62 (8.67)	76 (10.47)	52 (7.81)	333 (10.1)
<i>Talaromyces marneffeii</i>	70 (10.95)	41 (7.57)	73 (10.21)	53 (7.30)	48 (7.21)	285 (8.7)
Total ^b	639	541	715	726	666	3,287

^a Data presented as actual counts and percentages (%), ^b Total number of positive blood cultures per year (plus additional organisms not included in the table). CNS: coagulase-negative *Staphylococcus*

Figure 3. 2 The distribution of bloodstream pathogens during 2010-2014 period



Hospital admission: 235022

Blood cultures: 47,309

True positive cultures: 3,287

True positive culture rates: 6.9%

Pie charts showing combined data from 2004--2008. Proportions correspond to the organisms represented in the chart only (total data is shown in Table 3.4) and are shaded by *E.coli* (red), *Klebsiella* (green), nontyphoidal *Salmonella* and typhoidal *Salmonella* (marine), *Pseudomonas* and *Acinetobacter* and *Aeromonas* (peach), Gram-positive organisms(*Streptococcus* and *Staphylococcus*) (purple), fungi (*C.neoformans* and *T.marneffei*) (olive) and other bacteria (white blue).

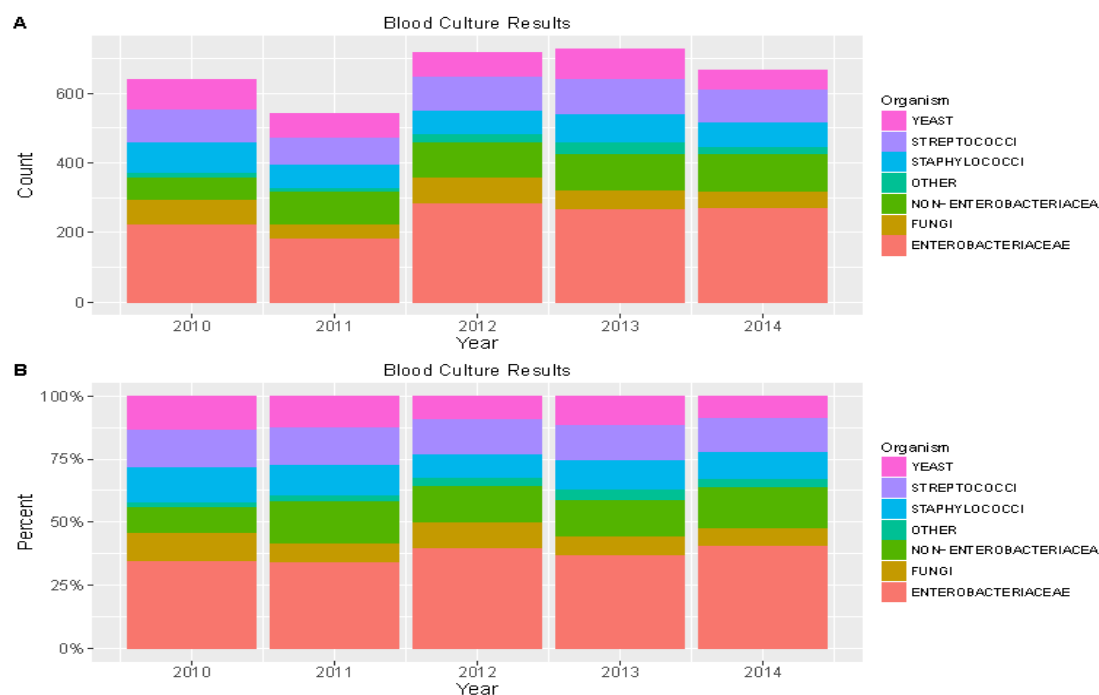
All eight of the Gram-negative cocci were *Neisseria meningitidis* serogroup b, and the eight Gram-positive bacilli included five *Listeria cytomonogenes*, one *Corynebacterium bovis*, one *Pleistophora* spp. and one *Nocardia* spp.

3.3.5.1 Gram-negative organisms

The Gram-negative bacilli were the most common group of pathogenic bacteria isolated (Figure 3.3). This group was comprised of the Enterobacteriaceae and the non-Enterobacteriaceae. *E. coli* was the most common bacterium isolated from blood in this hospital every study year, with a mean isolation rate of 20.9% (687/3,287) of all pathogens across the four years (Figure 3.2). There was an increase in the number isolated every year apart from 2011 over the study period (Figure 3.3). I additionally found that BSI associated with *E.coli* were significantly more commonly associated with community-acquired infections than hospital-acquired infections ($p<0.01$). Further, *E.coli* BSI were significantly more associated with the general wards than the ICUs, and more commonly resulted in good outcome ($p<0.001$) (Table 3.5).

The second most common pathogen was *Klebsiella* spp. in which *K. pneumoniae* predominated (254/256, 99.2%). The number of *Klebsiella* remained relatively consistent annually, and the proportion was three times less than *E.coli* (Table 3.4 % Figure 3.2). There was no significant association of *Klebsiella* bloodstream infection arising with community or hospital acquired infections or outcome (Table 3.5, Figure 3.3).

Figure 3. 3 The distribution of pathogens isolated from bloodstream infections by year (2010 -2014) at Hospital for Tropical Diseases.



Data is presented in real numbers (A) and percentages (B) for the main groups of pathogens causing BSI annually. Orange: *Enterobacteriaceae*; light green: filamentous fungi, green: non-*Enterobacteriaceae*; blue-green: unclassified organism; marine-blue: *Staphylococci*; purple: *Streptococci*; pink: yeast.

Table 3. 5 Causative pathogens by etiology of infection, outcome and ICU.

Pathogen	Total N(%)	Etiology of infection N=2,539				Outcome N=2,572				ICU admission N=2,325			
		CA	HA	X ²	p	Good	Bad	X ²	p	Yes	No	X ²	p
		2,129 (83.9)	410 (16.1)			1,667 (64.8)	619 (24.1)			1,091 (46.9)	1,234 (53.1)		
<i>E.coli</i>	687	465 (21.8)	67 (16.3)	6.3	0.01	395 (23.7)	96 (15.5)	17.9	<0.001	213 (19.5)	394 (31.9)	46.1	<0.001
<i>Klebsiella</i>	252	164 (7.7)	30 (7.3)	0.07	0.78	124 (7.4)	46 (7.4)	0.01	0.92	71 (6.5)	24 (1.9)	31.1	<0.001
<i>S.Typhi</i>	96	64(3.0)	6(0.7)	3.1	0.8	69 (4.1)	0 (0)	26.4	<0.001	4 (0.4)	91 (7.4)	72.6	<0.001
<i>Salmonella spp.</i>	164	118 (5.5)	9 (2.2)	8.1	0.004	77 (4.6)	34 (5.5)	0.75	0.38	32 (2.9)	34 (2.8)	0.07	0.79
<i>Acinetobacter</i>	101	25 (1.2)	60 (14.6)	192	<0.001	52 (3.1)	25 (4.0)	1.2	0.27	71 (6.5)	24 (1.9)	1.2	<0.001
<i>P.aeruginosa</i>	67	29 (1.4)	20 (4.9)	14.6	<0.001	22 (1.3)	20 (3.2)	9.1	0.002	34 (3.1)	12 (1)	13.7	<0.001
<i>Aeromonas</i>	91	58 (2.7)	6 (1.5)	2.2	0.13	39 (2.3)	20 (3.2)	1.4	0.23	49 (4.5)	33 (2.7)	5.6	0.01
<i>S.pneumoniae</i>	114	77 (3.6)	7 (1.7)	3.9	0.05	61 (3.7)	15 (2.4)	2.1	0.14	46 (4.2)	51 (4.1)	0.01	0.92
<i>S.suis</i>	123	104 (4.9)	2 (0.5)	16.6	<0.001	96 (5.8)	4 (0.6)	28.2	<0.001	93 (8.5)	29 (2.4)	44.4	<0.001
<i>Other</i>	225	150 (7)	17 (4.1)	4.7	<0.001	105 (6.3)	20 (3.2)	8.2	0.004	62 (5.7)	145 (11.8)	26.2	<0.001
<i>Streptococcus</i>													
<i>Enterococcus</i>	38	14 (0.7)	15 (3.7)	27.4	<0.001	18 (1.1)	9 (1.5)	0.5	0.46	18 (1.6)	14 (1.1)	1.1	0.28
<i>S.aureus</i>	272	170 (8)	41(10)	1.8	0.17	108 (6.5)	57 (9.2)	5.0	0.025	97 (8.9)	95 (7.7)	1.1	0.29
<i>Fungi</i>	637	464 (21.8)	39 (9.5)	32.6	<0.001	292 (17.5)	196 (31.1)	53.8	<0.001	75 (6.9)	44 (3.6)	13.0	<0.001

Table showed comparison data for 12 main pathogens in bloodstream infection by etiology of infection (2,539 cases), available outcome on discharge (2,572 cases) and by ICU/non-ICU care (2,572 cases). Pairwise comparison using X² test, p<0.05 was considered as significant.

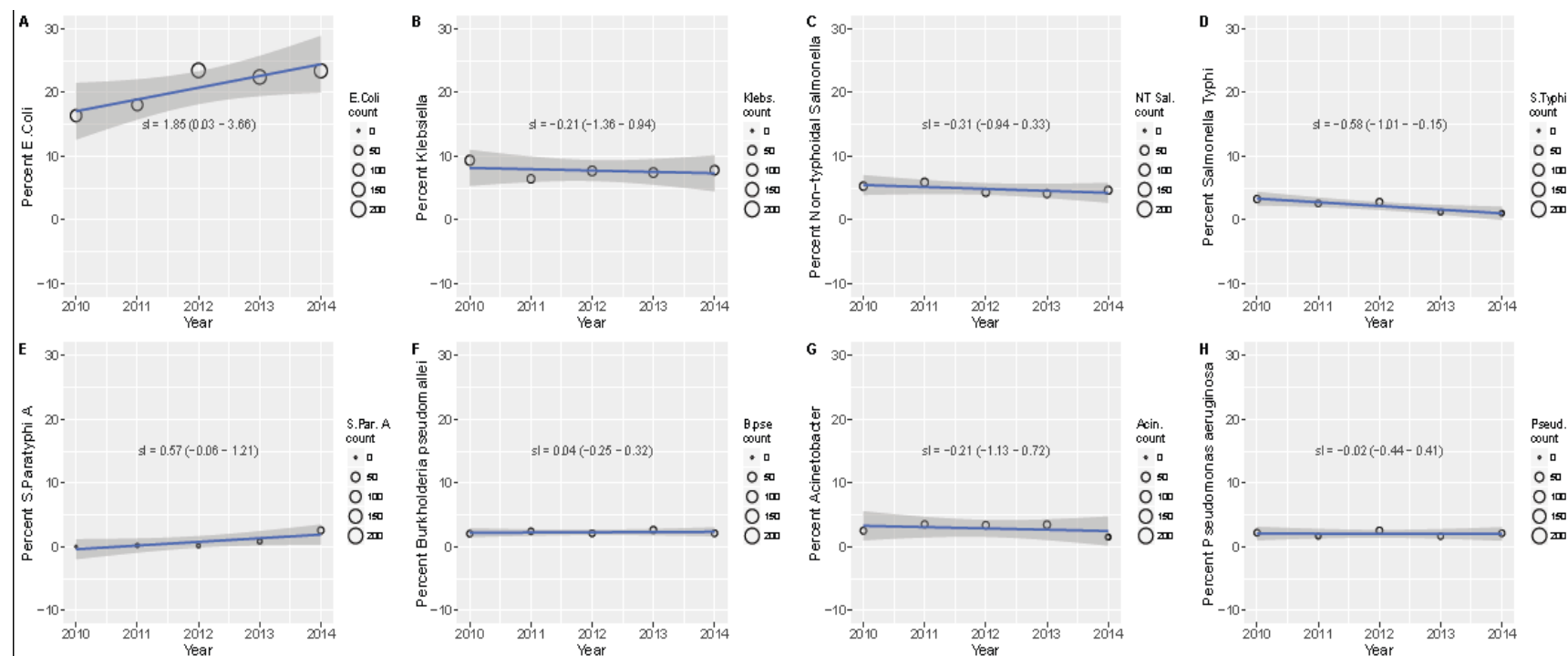
ICU category was determined based on the site of the blood culture collection.

However, *Klebsiella* was more commonly associated from patients in the general wards than in the ICU wards (71/1,091 vs 24/1,234; $p<0.001$) (Table 3.5).

Over the period of investigation 260 *Salmonella* were isolated, this included 96 *S. Typhi* and 164 non-typhoidal *Salmonella* (NTS). Typhoid fever, associated with *S. Typhi*, was found to be associated with a good outcome (full recovery on discharge) and less likely to arise in patients in the ICU ($p<0.001$) (Table 3.5). Notably, the number of NTS was greater than the number of *S. Typhi*. Further, and in comparable fashion to *Klebsiella* spp., the annual number of NTS isolated was relatively consistent (Figure 3.4). A notable characteristic of NTS cases is that 61.4% (97/158) of infections originated from HIV-infected patients. While *S. Typhi* exhibited an annual decline in number of isolations from 2010 to 2014, there was increase in the number of *S. Paratyphi A* (which causes a clinically indistinguishable disease from *S. Typhi*) isolated over the same period (Figure 3.4). None of those with a *S. Typhi* infection were HIV-infected.

Less common Gram-negative bacteria isolated from blood cultures were the non-fermenting bacilli. These included *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, and *Burkholderia cepacia*. A high proportion of *Acinetobacter* spp. and *Pseudomonas aeruginosa* (70.3% (71/94) and 50.7% (34/67), respectively) originated from the ICUs as apposed to the general wards ($p<0.001$). *Acinetobacter* spp. (most commonly *A. baumannii*) was the most frequently isolated non-fermenting rod (94/3,287; 2.9% of all BSI) and were more commonly associated with hospital-acquired infections and critical care ($p<0.001$) (Table 3.5).

Figure 3. 4 Time trends of the Gram-negative bacteria isolated from blood at HTD 2010-2014



Data was presented as percentage or actual count.

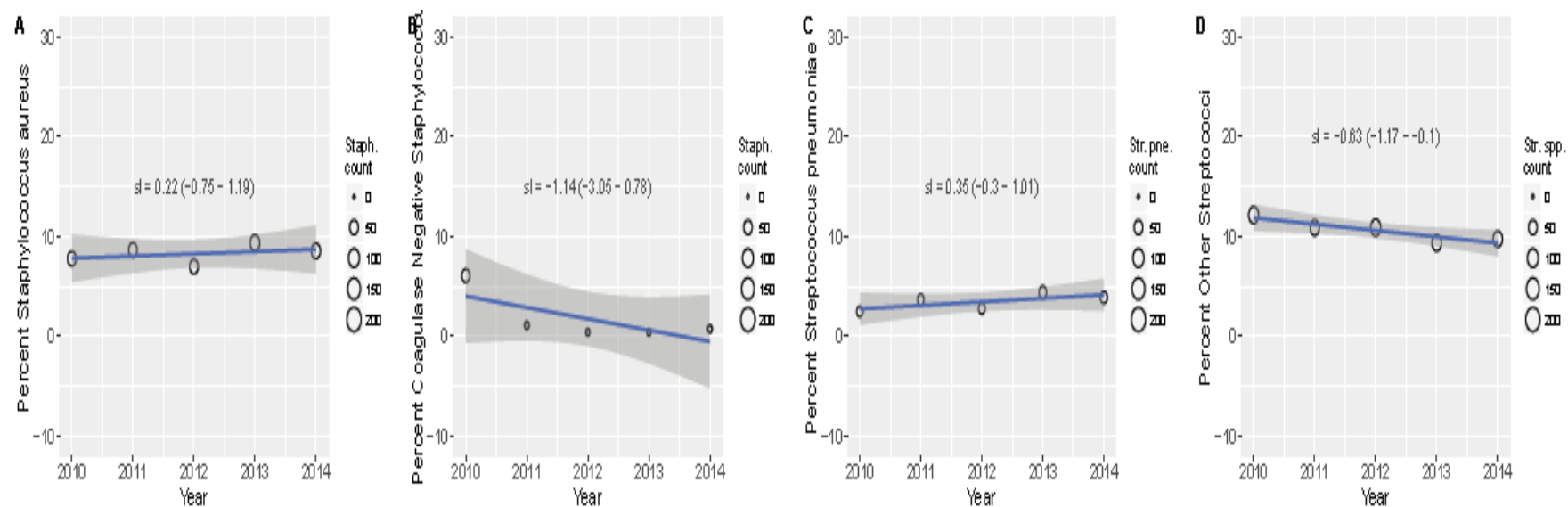
Klebsiella and Klebs: *Klebsiella* spp.; NT Sal.: Non-typhoidal *Salmonella*; S.Par.A: *Salmonella* Paratyphi A; Acin: *Acinetobacter* spp. ; Pseud. : *Pseudomonas aeruginosa*.

P. aeruginosa was also more commonly associated with hospital-acquired infections and those undergoing critical care ($p<0.001$) (Table 3.5). Additionally, *P. aeruginosa*, but not *Acinetobacter* spp., were more likely to be associated with a bad outcome ($p=0.002$). There was no significant increase or decline in the trend of *Acinetobacter* spp., *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, or *Burkholderia cepacia* over the study period (Figure 3.4)

3.3.5.2 Gram-positive organisms

The Gram-positive organisms represented 30% of all bacterial pathogens and 24.1% of all bloodstream isolations (Table 3.4, Figure 3.5). The most commonly isolated Gram-positive organisms were *Staphylococcus aureus*, *Streptococcus suis*, and *Streptococcus pneumoniae*, corresponding with 8.27% (272/3,287), 3.74% (123/3,287) and 3.47% (114/3,287) of all positive blood cultures, respectively (Table 3.4; Figure 3.5). The other *Streptococci* (including *S. agalactiae*, *S. pyogenes*, *S. salivarius*, and *S. gordonii*) represented only 6.85% (225/3,287) of all positive blood cultures. *S. aureus* and *S. pneumoniae* had showed an annual increasing trend over the five-year study period, whereas the coagulase negative *Staphylococcus* and other *Streptococcus* showed a decreasing trend over the same period (Figure 3.5); these changes with time were not significantly different. Over the study period there were 38/ 3,287 cases (1.2%) of *enterococcus* bloodstream infection. These infections were more likely to be associated with hospital-acquired infections than community-acquired infection ($p<0.001$) (Table 3.5). However, there was no difference in outcome or ICU admission in these *Enterococci* BSI ($p=0.46$ and $p=0.28$) (Table 3.5).

Figure 3. 5 Time trends of the Gram-positive bacteria isolated from blood at HTD 2010-2014



Data was presented as percentage or actual count.

Str.pneu: *Streptococcus pneumoniae*; Str.spp.: Other *Streptococcus spp.* (except *S.pneumoniae*)

3.3.5.3 Fungi

I found that *Cryptococcus neoformans* and *Talaromyces marneffe* (formerly known as *Pennicillium marneffe*) were the second and third most common pathogens isolated in the hospital over the period of investigation, (Table 3.4, Figure 3.2), representing 10.1% (333/3,287) and 8.7% (285/3,287) of the total positive blood cultures, respectively.

Despite being the second and third most commonly isolated pathogens, these organisms did not exhibit an increasing or decreasing trend over the five-year period. *Candida* were infrequently isolated, with only 19 cases throughout the five years.

3.3.6 Source of infection

Clinical data, describing a potentially associated source of infection were available for 321 infections only. The most common causes of primary infection were respiratory tract infections (174/321; 54.2%), followed by urinary tract infections (65/321; 20%), gastrointestinal infections (63/321; 19.7%), and wound infections (19/321, 5.9%) (Table 3.6).

Among the 174 BSI associated with respiratory tract infections, *S. pneumoniae* was the most common pathogen isolated, representing 18% (31/174 cases) of all BSI associated with respiratory tract infections. All respiratory tract infection associated bacteraemia cases caused by *S. pneumoniae* were associated with community-acquired infections and had a good outcome on discharge. Notably, *E.coli* and *Salmonella* were the second and the third more common organisms isolated from blood in those with a corresponding respiratory tract infection (Table 3.6). *S.aureus* and *K. pneumoniae* were cultured from blood of 13 and 12 individuals who had community-acquired

pneumoniae. Among the 13 *S.aureus* cases, five had a good outcome, one case was discharged to die at home, and the remainder transferred to other hospitals.

Table 3. 6 Infection source associated with bacteraemia in 321 patients attending HTD

Infection source	Number of pathogens*	1st	2nd	3rd	4th	5th
Respiratory	174	<i>S. pneumoniae</i> (31; 18%)	<i>E. coli</i> (25; 14%)	<i>Salmonella</i> (17;10%)	<i>S.aureus</i> (13; 8%)	<i>Klebsiella</i> (12; 7%)
Urinary tract infection	65	<i>E.coli</i> (46;71%)	<i>Klebsiella</i> (7; 11%)	<i>Streptococcus</i> (4; 2%)	<i>Salmonella</i> (3; 2%)	<i>S.aureus</i> (2; 1%)
Gastrointestinal	63	<i>E.coli</i> (18; 29%)	<i>Salmonella</i> (12; 19%)	<i>Klebsiella</i> (4; 6%)	<i>Campylobacter</i> (3; 5%)	Many organism
Skin and soft tissue	19	<i>S.aureus</i> (8; 42%)	<i>Klebsiella</i> (3; 16%), <i>Streptococcus</i> (3; 16%), coagulase negative <i>Staphylococcus</i> (3, 16%)			

* Data obtained from 321 patients with available source of BSI.

Members of *Enterobacteriaceae* (*E.coli*, *Klebsiella*, and *Salmonella*) were the most common organisms associated with BSI from those who also had urinary tract infections (UTI) and gastroenteritis. *E.coli* was isolated from blood of 71% (46/65) of cases with UTI/bacteraemia and almost 29% (18/63) of the cases with gastroenteritis/bacteraemia. Of those patients with an UTI/bacteraemia the majority were community acquired (46/65; 74%) and had good outcome (33/46; 72%). In those with gastroenteritis/bacteraemia the proportion of was 29% (18/63); 78% (14/18) of these were community-acquired infections and 78% (14/18) had a good outcome. Notably, there were three gastroenteritis/bacteraemia caused by *Campylobacter*, a microaerophilic organism isolated from an aerobic blood culture vial. The patients with a *Campylobacter* bacteraemia additionally had hepatitis and diarrhoea on admission. Lastly, *S.aureus* (42%; 8/19) was an important cause of bacteraemia in those with wound infections and skin abscesses; five out of eight were associated with community-acquired infections and had a good outcome (67%).

3.3.7 Antimicrobial susceptibility profiles

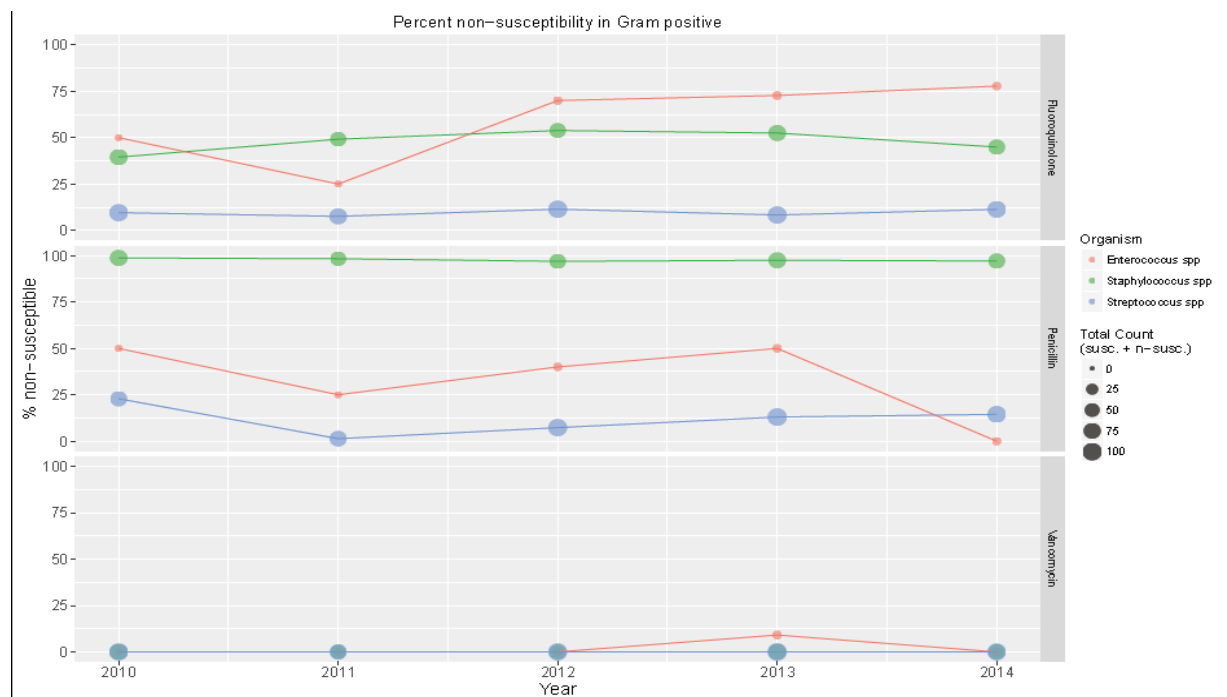
3.3.7.1 Gram-positive bacteria

Antimicrobial susceptibility testing was performed on isolation and the resulting data have been analysed for main antimicrobials used to treat Gram-positive infections. The resulting data were categorized into two groups: non-susceptible (resistant and intermediate resistance) and susceptible. Firstly, I examined the time trends for the *Staphylococci*, *Streptococci*, and *Enterococci* over the five-year period for the three main clinically relevant antimicrobials: penicillins, fluoroquinolones (ciprofloxacin or levofloxacin when available), and vancomycin (Figure 3.4). Overall, there was no significant change in the dynamics of susceptibility against these antimicrobials apart

from the enterococci, which had an increase in resistance against fluoroquinolones and an increase in susceptibility for penicillin.

The *S.aureus* isolated over the study period (n=272) were characterized with high proportion of resistance against penicillin; 99% (269/272) of *S.aureus* isolated from blood were resistant to penicillin. Besides three main antimicrobial listed above, available susceptible results for *S.aureus* were also found from clindamycin, co-trimoxazol and rifampicin. Non-susceptibility to other anti-Staphylococcal agents such clindamycin, ciprofloxacin, and co-trimoxazole were 72,0% (196/272), 44.5% (121/272), and 39.3% (107/272), respectively. Methicillin resistant *S.aureus* (MRSA) accounted for 45.6% (124/272) of all isolated *S.aureus* during 2010-2014 (ranging from 38.2% to 57.3% per year). Moreover, MRSA were associated with high rate of non-susceptibility against of common antimicrobials. MRSA were significantly more likely to be non-susceptible to clindamycin, ciprofloxacin, and co-trimoxazole than in methicillin-susceptible *S.aureus* (MSSA) ($p<0.001$ for clindamycin and ciprofloxacin, $p=0.003$ for trimethoprim-sulfamethoxazol). Non-susceptibility against to rifampicin was less common, arising on only 16/272 infections (5.9%). There were no *S.aureus* isolated that were non-susceptible to vancomycin, the maximum MIC against vancomycin (by E-test) over the study period was 2.0µg/ml.

Figure 3. 6 Annual non-susceptibility patterns of the three main Gram-positive species to penicillin, vancomycin, and fluoroquinolones at HTD, 2010-2014.



Data presented in percentage on X- axis; estimated total count every year was presented by the size of the dot. *Enterococcus sp.* : red line (dot); *Staphylococcus spp.* : green line (dot); *Streptococcus sp.*: blue line (dot)..

S. pneumoniae was the most resistant antimicrobial member of the *Streptococci*. Very high non-susceptibility rates were recorded against erythromycin, clindamycin, and co-trimoxazole, identified in 87.7% (100/114), 80.7% (92/114) and 79.8% (91/114) of organisms, respectively. In contrast, 91% (104/114) *S. pneumoniae* were susceptible to penicillin. The MIC₅₀ and MIC₉₀ against penicillin were 0.25 µg/ml and 0.75 µg/ml, respectively. All *S. pneumoniae* were susceptible to vancomycin. *S. suis* was also commonly isolated from febrile patients, however this organism was susceptible to all clinically relevant antimicrobials, including penicillin, ceftriaxone, and vancomycin. The MIC₅₀ and MIC₉₀ against penicillin for *S. suis* were 0.03 µg/ml and 0.06 µg/ml, respectively, which were lower than those recorded for the pneumococci. The MIC₅₀ and MIC₉₀ against ceftriaxone in the isolated *S. suis* were also low (0.01 µg/ml and 0.1 µg/ml, respectively). However, in a comparable fashion to *S. pneumoniae*, *S. suis* exhibited a high rate of non-susceptibility against macrolides; 48% (59/123) were non-susceptible to erythromycin and 41% (50/123) were non-susceptible to clindamycin. The isolated beta hemolytic *Streptococci* included *S. agalactiae* (52.3%; 34/65), *S. pyogenes* (32.3%; 31/65), *S. dysgalactiae* (15.4%; 10/65). These organisms were all susceptible to penicillin, ceftriaxone, vancomycin, and rifampicin. However, 80% (52/65) of the beta-hemolytic *Streptococci* were non-susceptible to tetracycline. The non-susceptibility rate to erythromycin, levofloxacin, and co-trimoxazole were 47% (31/65), 25% (16/65) and 15.4% (10/65), respectively.

From the 38 isolated enterococci, 66% (25/38) were non-susceptible to fluoroquinolones (either ciprofloxacin or levofloxacin). None of the enterococci were susceptible to erythromycin, only 5/38 (13.1%) cases were susceptible to tetracycline. The overall resistance rate to penicillin and ampicillin was 31.6% (12/38) and 34.2% (13/38), respectively. Among all the enterococcus species, *E. faecium* was the species

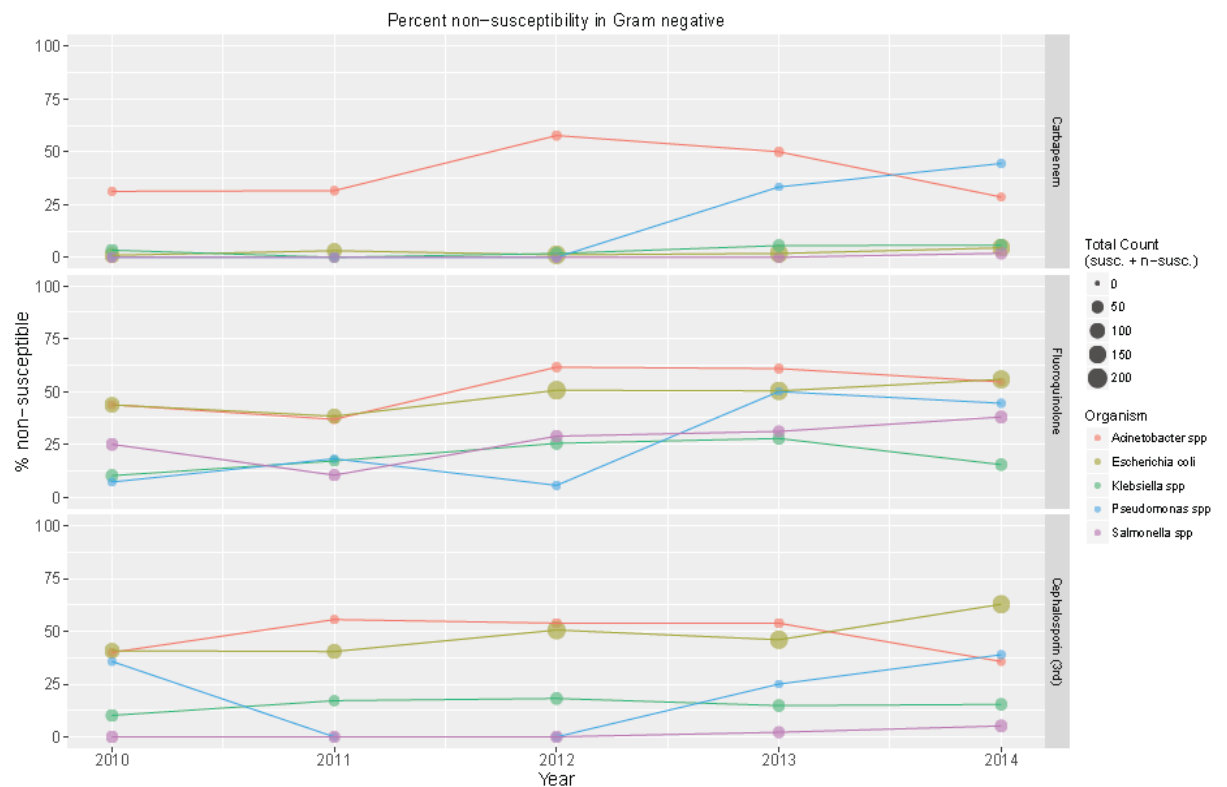
with the broadest range of resistance to antimicrobials with 82% (9/11) of organisms being resistant to ampicillin, and 73% (8/11) being resistant to penicillin. Resistance against vancomycin was found only in one *E. faecium* isolate, this isolate had MIC against vancomycin of >256µg/ml, and was non-susceptible to all other antimicrobials tested, apart from linezolid. Notably, *E. faecalis* were more susceptible to beta-lactams than *E. faecium*, with only 31.8% (7/22) and 22.7% (5/22) of organisms being resistant to penicillin and to ampicillin, respectively. However, all *E. faecalis* were non-susceptible to tetracycline and erythromycin; none were resistant to vancomycin, all had MICs <2µg/ml.

3.3.7.2 Gram-negative bacteria

Antimicrobial susceptibility testing was performed on isolation and the resulting data analysed for the main antimicrobials used to treat Gram-negative infections. The resulting data were categorized into non-susceptible (resistant and intermediate resistance) and susceptible as before. I examined the time trends for *Acinetobacter* spp., *E. coli*, *K. pneumoniae*, *Pseudomonas* spp., and *Salmonella* spp. over the five-year period against carbapenems, fluoroquinolones, and third generation cephalosporins (Figure 3.6). Over this period the general trend was a decrease in susceptibility to all these commonly used antimicrobials. The proportion of non-susceptibility against third generation cephalosporins increased from 40.8% (42/103) in 2010 to 60.8% (95/156) in 2014, with a median annual increase of 4% (Figure 3.6). Comparatively, the proportion of *E. coli* that were non-susceptible to fluoroquinolones also increased annually, rising from 42.7% (44/103) in 2010 to 54.5% (85/156) in 2014. There was also an increasing trend of non-susceptibility to carbapenems from 1.0% (1/103) in 2010, to 3.8% (6/156) in 2014. The majority of *E. coli* isolates that had decreased susceptibility to carbapenems were intermediately resistant or resistance to ertapenem, a first generation

carbapenem. However, there was no antimicrobial non-susceptible profile for *E.coli* that was associated with poor outcome ($p>0.05$)(Table 3.7). Extended spectrum beta lactamase (ESBL) activity was recorded in 42.4% (291/687) of all isolated *E. coli*. AmpC lactamase activity was recorded in 0.8% (6/687) of all isolated *E. coli*.

Figure 3. 7 Annual non-susceptibility patterns of the five main Gram-negative species to carbapenems, fluoroquinolones, and third generation cephalosporins at HTD, 2010-2014.



Data presented in percentage on X- axis; estimated total count every year was presented by the size of the dot. *Acinetobacter* spp. : red line (dot); *Escherichia coli* : olive line (dot); *Klebsiella* spp. : green line (dot); *Pseudomonas* spp.: blue line (dot); *Salmonella* spp.: purple line (dot)

Table 3. 7 Susceptibility profile of 491 *E.coli* against 3rd cephalosporin, carbapenem and fluoroquinolone in different outcome groups

Outcome	3 rd cephalosporin		Carbapenem		Fluoroquinolone	
	n (%)		n (%)		n (%)	
	S	NS	S	NS	S	NS
	395 (80.4)	96 (19.6)	395 (80.4)	96 (19.6)	375 (76.4)	116 (23.6)
Good	213 (53.9)	50 (52.1)	386 (97.8)	92 (95.8)	200 (53.3)	64 (55.2)
Bad	182 (46.1)	46 (47.9)	9 (2.3)	4 (4.2)	175 (46.7)	52 (44.8)
X^2	0.1		1.07		0.03	
p	0.7		0.3		0.86	

Data obtained from 491 *E.coli* that had available antimicrobial profile for investigated antimicrobials. Pairwise comparison using X^2 test, $p < 0.05$ was considered as significant.

S: susceptible; NS: non-susceptible

In comparison to *E. coli*, the isolated *Klebsiella* were less commonly resistant to third generation cephalosporins and fluoroquinolones. The annual prevalence of resistance against third generation cephalosporins ranged from 10% to 18%, while the annual prevalence of resistance against fluoroquinolones ranged from 7% to 22%. However, comparable to *E. coli*, the isolated *Klebsiella* had an annual increase in resistance to carbapenems; none of the *Klebsiella* were resistant to carbapenems in 2011 and 5.7% (3/52) were resistant in 2014.

Salmonella

Among the 96 *S. Typhi* isolated, an MDR phenotype (resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole) was recorded in 13 (14%) isolates, this proportion became fewer with time. Almost half (42/96; 44%) *S. Typhi* were non-susceptible to nalidixic acid. However, all isolated *S. Typhi* were susceptible to fluoroquinolones and third generation cephalosporins. The HTD microbiology laboratory did not test susceptibility for ampicillin and chloramphenicol routinely for the NTS; therefore data was not available for these two antimicrobials. Overall, resistance against third generation cephalosporins was rare with only 4/126 (3%) of NTS not susceptible to ceftriaxone. Non-susceptibility to the fluoroquinolones, ciprofloxacin and to ofloxacin was identified in 26/126 (21%) and 6/126 (5%) of organisms, respectively. Four NTS organisms were non-susceptible to both ciprofloxacin and ofloxacin.

Acinetobacter

The isolated *Acinetobacter* spp. generated a similar susceptibility pattern for cephalosporins, carbapenems, and fluoroquinolones. The non-susceptible trend tended to increase from 2011 to 2013 then decline in 2014. Moreover, *Acinetobacter* was the

most non-susceptible organism against many antimicrobial classes (3rd cephalosporin, carbapenem, fluoroquinolone...) found in bloodstream infections. 43% (43/94) *Acinetobacter* had non-susceptible profile to three main classes: 3rd cephalosporin (ceftazidime), fluoroquinolone (either ciprofloxacin or levofloxacin) and carbapenem (imipenem and/or meropenem), and only susceptible to colistin. Ceftazidime nonsusceptibility was recorded in 48/94 (47.9%) cases while cefepime nonsusceptibility was in 50 cases (53%). Non-susceptibility to imipenem and meropenem were high at 56.4% (53/94) and 44.7% (42/94). Trimethoprim-sulfamethoxazole was ineffective in 48% (48/94) of *Acinetobacter*.

Pseudomonas

Pseudomonas were the only Gram-negative organisms that increased in the proportion of non-susceptible isolates to multiple agents over the five-year period. Non-susceptible *pseudomonas* to ceftazidime and carbapenem increased from 0% (0/18) in 2012 to 50% (7/14) in 2014. 17.4% (12/69) MDR *Pseudomonas* (mostly *P. aeruginosa*) were isolated in 2013 and 2014 (4 isolates in 2013 and 8 isolates in 2014).

3.3.8 The aetiology of common organism and common resistant mechanism

Community or nosocomial

The six most common BSI pathogens were stratified by their association with community or hospital acquired infection. *Acinetobacter* and *Pseudomonas* were significantly more associated with hospital acquired BSI than community acquired BSI ($p < 0.001$) (Table 3.5). In contrast, *E. coli*, *Salmonella*, *Streptococcus*, and *S. aureus* were significantly more associated with community acquired BSI than in hospital acquired BSI ($p < 0.05$ for *Salmonella*, $p < 0.001$ for *Streptococcus* and *S. aureus*)

(Table 3.5). *Klebsiella* were the only common pathogens that were not significantly associated with community acquired or hospital-acquired BSI (Table 3.5).

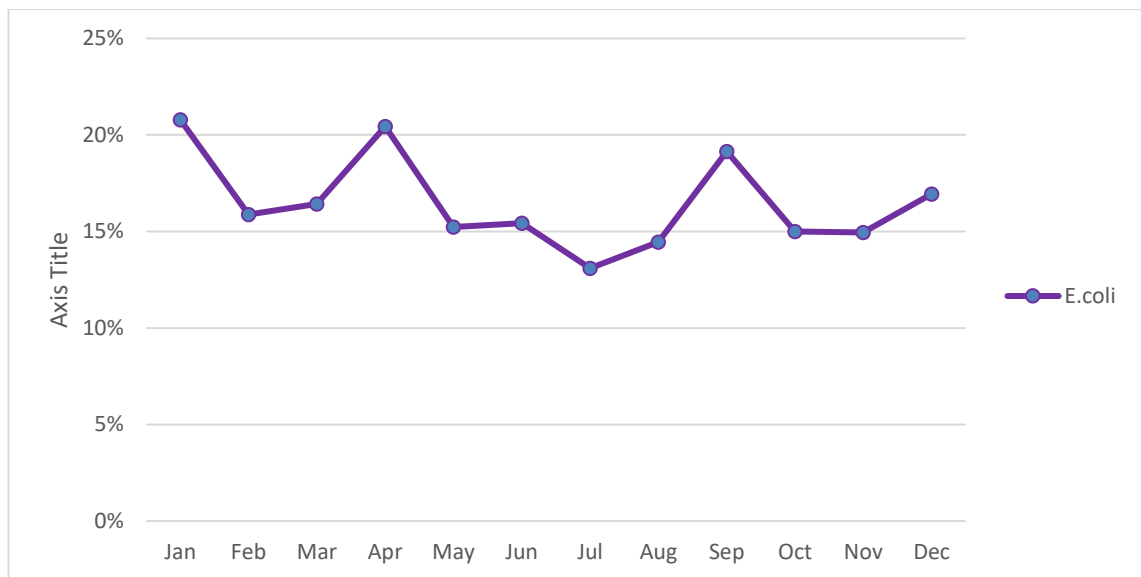
Common resistant mechanisms

I next analysed the prevalence of ESBL in the *Enterobacteriaceae* and MRSA, the two most clinical relevant resistant mechanisms associated with community/hospital-acquired infections. ESBL activity was determined in 178/1,583 (9.4%) of the community-acquired BSI organisms and 45/441 (9.8%) of the hospital-acquired BSI organism. Therefore, the ESBL activity rate was not significantly difference between two groups ($p=0.72$, Fisher exact test). However, MRSA was found more commonly associated with hospital-acquired BSI than in community-acquired BSI (5.5% vs. 3.5%, respectively) ($p=0.06$, Fisher exact test).

3.3.9 Seasonal variation

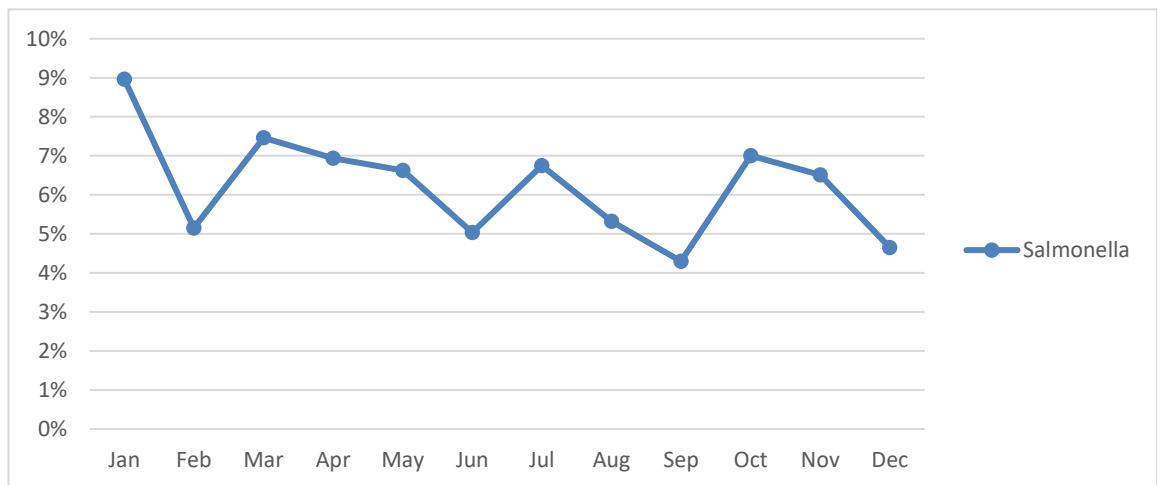
In the south of Vietnam, there are two main seasons, a rainy season and dry season. The rainy season runs from April to October every year while dry season is in the intervening months. I analysed the seasonal distribution of two key organisms (*E.coli* and *Salmonella*). I observed that *E. coli*, the most common BSI pathogen had two seasonal peaks, one in April and one in September. This observation equated with more BSI associated with *E.coli* at the beginning and at the end of the rainy season. I found that BSI caused by *Salmonella* typically peaked between January March, July and October every year and declined at the end of the year. Therefore, the peak of *Salmonella* BSI infection mostly happened during rainy season (from April to October) (Figure 3.8 and Figure 3.9).

Figure 3. 8 Seasonal variation of *E.coli* in bloodstream infection in HTD during 2010-2014.



Plot showing incidence of bloodstream infection due to *E.coli* among all pathogens every month during 2010-2014 period.

Figure 3. 9 Seasonal variation of *Salmonella* bloodstream infection in HTD during 2010-2014.



Plot showing incidence of *Salmonella* bloodstream infection among all pathogens every month during 2010-2014 period.

3.4 Discussion

In HTD, the annual positive rate of blood culture over the defined study period was 7% (6.1%-7.3%) which was lower than other hospitals in Asia and other continents (Ahmed et al., 2017; Crump et al., 2015; Orsini et al., 2012; Phetsouvanh et al., 2006; Sang Oh et al., 2015). An explanation for lower culture frequency may include; only one blood vial was collected per episode, under filled blood bottle for culture, and the pre-consumption of antimicrobials. Many studies have shown that sensitivity of blood culture can reach up to 60% if more than 20ml of blood is inoculated into the bottle (Art, 2016) or at least recommended to use more than one bottle for blood culturing from adult patients (Lee et al., 2007). Additionally, although there was no information recorded about pre-antimicrobial consumption, I predict a high proportion of cases that receiving an antimicrobial before blood culturing, which is the most likely explanation for the low rate of positive blood cultures. Recent guidelines have suggested that using resin-media, which can absorb antimicrobials in the blood, and collecting enough blood volume can improve the rate of pathogens recovery from blood culture. Moreover, applying molecular method to improve the diagnostic rate and identify common resistant mechanisms may also be a promising solution.

The low overall contamination rate of blood culture was low (1.2% (565/ 47,309)), which reflected good blood collection technique of the nursing staff in this hospital. However, there may have been potential errors on the decision of contamination because judgment was made through agreement between laboratory staff and an attending doctor based on the result of one blood culture sample for each infection episode. I strongly recommended that a set of two blood culture collections should be performed routinely in HTD, each culture from a different venous site so that contamination can be ruled out.

One distinctive contaminant in HTD was organisms belonging to the *Burkholderia cepacia* complex which accounted for 58% (329/565) of all contaminants. The decision of defining *Burkholderia cepacia* was for every individual case because this group of organisms can be defined as a pathogen in some situations, such as with the contamination of intravenous fluids (Doit et al., 2004; T. Singhal et al., 2015). In this context, there were 32 *B. cepacia* cases which were considered as to be associated with BSI, because they were collected from culture of HIV infected patients or those admitted to the ICU. It is known that *B. cepacia* complex has the capacity to survive in some disinfectants, therefore correct blood collection techniques should be re-taught annually for ICU staff and those working with HIV infected patients. Some skin preparation techniques, such as the initial specimen diversion technique, has been shown to reduce contamination by up to 30% in a study from Canada (Binkhamis and Forward, 2014).

Coagulase negative *Staphylococcus* (CNS) were the second most common contaminant in HTD with rate of 36.5% (206/565) of all contaminated blood cultures. Most of these contaminated cultures were taken from the children's ward and HIV ward. CNS is a common contaminant of blood culture in many reports (Gregson and Church, 2007; Kanoksil et al., 2013; Kiertiburanakul et al., 2012).

In this study, one third of BSI (33.2%, 1,091/2,587) originated from the ICU wards and one fifth (22%, 962/2,587) were from HIV-ward. These two wards possessed the highest proportion of BSI in HTD over the five-year investigation. In many BSI studies, critical care units account for very high proportion of BSI because they receive severely ill patients, patients with underlying diseases, or those with the potential risk of hospital-acquired infections (Barnett et al., 2013; Timsit et al., 2014). In the epidemic areas a high prevalence of HIV infection, such as Southeast Asia and Africa, a large

proportion of BSI originated from HIV-infected patients. My study found that 22% (952/2,587) BSI were HIV-seropositive patients, which was considerably lower than data obtained from a meta-analysis from Africa (53.5%, 1,217/2,273)(Reddy et al., 2010). An explanation for this observation is the availability of antiretroviral therapy and better nutrition care in Vietnam than in many African countries.

I found that fatality rate in this study was 24% (619/2,572), which included death in the hospital and moribund. A recent publication originating from a tertiary hospital for infectious diseases in Northern Vietnam showed a higher fatality rate of 28.9% (138/477) of BSI during 2011-2013 (Dat et al., 2017). However, a study of BSI from an African hospital, which included HIV and non-HIV infected patients had lower fatality rate of 14% in 2009. Notably, this study demonstrated a significantly reduction of mortality rate from 39% in 1997 to 14% in 2009 ($p<0.001$), after the introductions of ARTs (Feasey et al., 2014). Although there was a decline in fatality outcome in HTD from 26.3% (183/696) in 2012, to 20.5% (144/703) in 2013, this declining trend was not as pronounced as in the African hospital study and ARTs were available in Vietnam during the entire study period.

In my study, patient population had median age of 42 (IQR 29-56) years which was younger than BSI population in the north of Vietnam (48; IQR 36-60)(Dat et al., 2017). However, an even younger population admitted to hospital due to BSI was found in Southeast Asia: mean age 31 years old (IQR 27-38)(Jay K Varma et al., 2010) ; and Africa, 38 years old (IQR 14-96) in Tanzania(John A. Crump et al., 2011). These countries are all under-developed nations with an on-going HIV epidemic. In contrast, BSI surveillances in developed countries generally has an older patient population (Skogberg et al., 2012; Wisplinghoff et al., 2003; Yahav et al., 2016). In comparison to this study, healthcare-associated and nosocomial BSI have been found more common in

older patients (Kaye et al., 2014; Mayr et al., 2014; Yahav et al., 2016). The elderly have been identified as being at risk of nosocomial infection in the guidelines for management of BSI (P. Dellinger et al., 2013). My study emphasizes the importance of clinical assessment for nosocomial BSI in patients aged over 60 years who are admitted in HTD.

Males were the predominant sex in my study (63.9%; 2,102/3,287). Similar findings have been reported in studies of BSI in Vietnam, Asia, and other developing countries (Dat et al., 2017; Vlieghe et al., 2012)(Wisplinghoff et al., 2004)(Kiertiburanakul et al., 2012).

One limitation of my study was that data about age and sex of all patients in hospital during 2010-2014 were unable obtained. Therefore, it was impossible to clarify the differences of age and sex distribution between BSI patients with other types of hospital admission.

The pathogen type in BSI is largely dependent on the characteristics of the patient population within the hospital. HTD is a non-surgery hospital which received both HIV and non-HIV infected patients. Therefore, fungi accounted for 19.4% (637/3,287) of total BSI in HTD; 87% of these 637 cases originated from HIV-infected patients. Fungal infections in HTD have been intensely researched at OUCRU for several years, mainly focusing on *Cryptococcus neoformans* and *Talaromyces marneffe* (Ho Dang Trung et al., 2012; Le et al., 2011). The proportion of fungi in BSI at HTD Vietnam was lower than that found (24%) fungi in a cohort study about BSI in a similar hospital in Thailand (Kiertiburanakul et al., 2012). I found that bacteria were the most common group of BSI pathogens at HTD accounting for 80.6% (2,650/3,287) of all infections; Gram negatives were a higher proportion than Gram positives (66.4% (890/2,650) vs. 33.6% (1,760/2,650)). Dat *et al.* performed a BSI study in the north of Vietnam that showed a similar proportion of Gram-negative (69%) and Gram-positive (31%) as

found here. The proportion of Gram-negative bacteria in my study was lower than other studies in Southeast Asia. BSI in Thailand was associated with 72% Gram-negative and 28% Gram-positive bacteria (Kiertiburanakul et al., 2012); Cambodia was 78.9% Gram-negative and 21.1% Gram-positive organism (Vlieghe et al., 2013); Laos was 77.2% Gram-negative and 22.8% Gram-positive bacteria (Phetsouvanh et al., 2006) . However, a meta-analysis of 61,327 BSI cases in Africa, found a lower proportion of Gram negative than reported here: 58.2% Gram-negative and 33.4% Gram-positive bacteria (Reddy et al., 2010).

In this study, I found *E.coli* was the largest cause of BSI, which is comparable to many other studies of septicemia globally (Deen et al., 2012; Kang et al., 2012; Kanoksil et al., 2013; Serefhanoglu et al., 2009). *E.coli* was also the only bacteria that exhibited an increasing trend in the period 2010-2014. This increase may be explained by the an additional increase in the number of patients with severe hepatitis and ascites admitted to this hospital; *E.coli* was the most common pathogen causing septicemia in this patient group. Additionally, I found a higher proportion of NTS than *S. Typhi* between 2010-2015. This is different from a similar study conducted at HTD 1990s and 2000s in which *S. Typhi* was the predominant bloodstream pathogen (Nga et al., 2012). In Africa, NTS have been found to be more common in HIV-infected patients than in immunocompetent hosts (Biggs et al., 2014; Feasey et al., 2012).

Worryingly, I report a high proportion of MRSA and ESBL-producing bacteria associated with community and hospital acquired infections. In the recent HTD antimicrobial guidelines, third generation cephalosporins and oxacillin are the drug of choice for community infections for Gram-negative bacterial infections and *Staphylococcus aureus*, respectively. This study suggests that clinicians should request blood and other infected samples for culture so that resistant organism can be intensified

and treatment can be modified as quickly as possible. Moreover, the HTD antimicrobial guidelines should be revised frequently with the updated antimicrobial susceptibility data.

Although there was only a single VRE isolated over this five-year period, the presence of this dangerous pathogen in a hospital setting in Vietnam is concerning. Since the discovery of VRE in 1987, this organism has spread internationally (Willems et al., 2005). There are very few antimicrobials that are able to treat VRE, these include the expensive treatments, linezolid, teicoplanin, and daptomycin. Thus, I suggest that infection control practices should be applied to minimize the spread of VRE and other MDR bacteria.

Notably, *Acinetobacter* and *Pseudomonas* were the only two organisms that were found to be significantly more common in hospital-acquired infections than community-acquired infections ($p < 0.05$). High resistance levels to several broad-spectrum antimicrobials used in the ICUs characterized these organisms. Resistance to third generation cephalosporins, carbapenems, and fluoroquinolones here were as high as numbers reported in Asia, Europe and USA (Al-Mously, 2013; Arnoni et al., 2007; Kang et al., 2003; Mudau et al., 2013; Wisplinghoff et al., 2004). I would like to emphasize the significance of the high prevalence of resistance to common antimicrobials because the last drug against HTD is now colistin. I suggest that an antimicrobial stewardship and better infection control measures should be applied more strictly at HTD. These measures are particularly important in the ICU ward to minimize the spread of these MDR bacteria within the ward and to other parts of the hospital.

I observed an increased trend of AMR, which was particularly apparent for ESBL producing *E.coli*. The proportion of ESBL producing *E.coli* increased and there was no

association between infections arising in the hospital or the community. These data suggest that the largest reservoir of MDR is in the community, which is very alarming for the large population of HCMC. The data presented in this chapter have been used to build the first ever antimicrobial treatment guidelines in HTD. The first version was published in 2015, and contained recommendations for antimicrobials for community and hospital-acquired infections, including BSI. The suggested empirical antimicrobial therapy for BSI was determined from the common pathogens and their susceptibility patterns identified from this study. Moreover, this work continued and a new revision will be released at the end of 2017.

3.5 Two cases of non-toxigenic *Vibrio cholerae* non-O1, non-O139 bacteremia in Ho Chi Minh City

3.5.1 Case one

A 63-year-old female patient was admitted to HTD in June 2013. She recalled a four-week history of fatigue with the loss of appetite, and she had developed jaundice with fever in the week prior to admission. The patient had a history of hypertension, but no previous history of liver disease. On admission, she was fully conscious, afebrile and hemodynamically stable, her respiratory rate was 20 breaths per minute. She had severe icterus, palmar erythema, peripheral edema, and her liver and spleen were not palpable. She had detectable ascites (Grade 2) but without abdominal tenderness or portal vein thrombosis. The patient's initial laboratory results are shown in Table 3.1 and her viral hepatitis serology results were as follows; antiHAV (IgM) - negative, antiHBV - negative, antiHBc (IgM) - negative, antiHCV - negative, and HBsAg - positive, with a quantitative HBV PCR blood result of 1×10^6 copies/ml, (Abbott Real-time HBV Kit, USA). The initial prescribed treatments were entecavir (0.5mg once a day), rabeprazole (20mg twice a day), BDD (25mg thrice a day), furosemide (25mg once a day), and losartan (50 mg once a day). On the third day of admission, the patient's temperature peaked at 40°C with associated chills, nausea and dizziness, but without diarrhea or abdominal pain. Her procalcitonin was elevated at 0.72 ng/ml. A bacterial infection was suspected, and 2g/day of intravenous ceftriaxone was added to the medications. An aerobic BACTEC bottle was taken for blood culture and incubated in a BACTEC 9240 system. It became positive after 12 hours. A Gram stain on the positive BacT/Alert revealed small curved Gram-negative bacilli, which were sub-cultured onto blood and MacConkey agar plates. The colonies displayed hemolysis on the blood agar plates and were oxidase positive. API20E and VITEK2 identification (bioMérieux, France) confirmed the organism to be *Vibrio cholerae*. Slide agglutination tests with polyvalent O1 and O139 antisera were negative. The isolate was resistant to trimethoprim-

sulfamethoxazole and tetracycline and susceptible to chloramphenicol, ofloxacin, ciprofloxacin, azithromycin, and ceftriaxone. The patient was became afebrile after two days of ceftriaxone, yet the antimicrobial was continued, with the other treatments, for an additional eight days. The patient was ultimately discharged after 24 days of hospitalization.

Table 3. 8 Initial laboratory test results of two patients with *Vibrio cholerae* non-O1, non-O139 bacteremia

Laboratory test	Normal range	Patient 1	Patient 2
White cell count ($\times 10^3$ cells/ μ l)	6-10	7.37	1.75
Polymorphonuclear cell (%)	49.6-71.3	58.4	83.2
Lymphocyte (%)	27.8-42.2	34.4	14.2
Monocyte (%)	0.2-4.3	18.2	0.8
Red cell count ($\times 10^6$ cells/ μ l)	4.7-5.4	4.0	3.9
Platelet ($\times 10^3$ cells/ μ l)	201-324	160	37
AST (UI/l)	<37	2983	121
ALT (UI/l)	<40	3004	56
Total bilirubin (μ mol/l)	0-17	334.5	2.1
Direct bilirubin (μ mol/l)	0-4.3	272	3.2
Albumin (g/l)	35-50	27.9	29.6
Prothrombin time (s)	11-13.5	23.9	18.1
TP (%)	70-130	35	55
International Normalized Ratio	0.85-1.15	2.22	1.48

AST: aspartate aminotransferase, ALT: alanine aminotransferase, TP: prothrombin time

3.5.2 Case two

In July 2013 a 73-year-old man was admitted to HTD with fever and confusion. He had been diagnosed with severe cirrhosis due to hepatitis C infection in a private healthcare facility four years previously, and was actively receiving an unspecified treatment regime. He had been unwell for four days with fever and constipation. Initial examination on the day of admission to hospital revealed the man to be thin, pale, and icteric, with peripheral edema and spider angiomas. His pulse was 97 bpm, with a blood pressure of 140/80 mmHg, and a respiratory rate of 24 breaths per minute. He was febrile with a temperature of 39°C. His mental state was confused, somnolent and he had amnesia. A chest X-ray suggested the patient had pneumonia and an abdominal examination showed marked ascites with tenderness. An abdominal ultrasound revealed large amount of ascitic fluid and splenomegaly. A bacterial infection was suspected; therefore the patient was prescribed 2g/day of intravenous ceftriaxone, along with metronidazole (250 mg/day), furosemide (25 mg/day), and lactulose.

A yellow sample of ascitic fluid was drawn, which was rivalta test negative, and negative for bacteria by Gram staining on microscopy. The fluid had 629 leucocytes/mm³ (86% neutrophils and 14% lymphocytes), 1,000 erythrocytes/mm³, 9 g/L of protein, and 5.8 g/L of albumin. The ascitic fluid was cultured on blood agar and MacConkey agar and an aerobic BacT/Alert tube was taken for blood culture. Gram-negative curved bacilli were from both the blood and the ascitic fluid isolated after overnight incubation. The organism in both samples was identified as *V. cholerae* and neither agglutinated with O1 and O139 antisera. Antimicrobial susceptibility testing demonstrated the organism was susceptible to all antimicrobial tested (ampicillin, chloramphenicol, ciprofloxacin, ceftriaxone, ofloxacin, trimethoprim-sulfamethoxazole,

and tetracycline). The patient became afebrile after two days of ceftriaxone, but was transferred to another hospital for surgical intervention after being diagnosed with bleeding of the upper gastrointestinal tract.

For confirmation of the microbiological identification, DNA preparations from the isolates from both patients were subjected to established PCR amplification methods targeting the rRNA intergenic spacer region of *V. cholerae* (Chun et al., 1999), the cholera toxin (CT) gene *ctxA*, the O1 O-antigen, and the O139 O-antigen (Choopun et al., 2002). A toxigenic *V. cholerae* strain previously cultured from stool of diarrheal patient was used as a control for the assays. All three of the isolates (two from blood and one from ascitic fluid) were PCR amplification positive for the rRNA intergenic spacer, confirming their microbiological identification as *V. cholerae*. All of isolates were PCR amplification negative for the O139 antigen, the O1 antigen, and the *ctxA* toxin gene. Hence we concluded the isolates to be non-toxigenic, non-O1, non-O139 *V. cholerae*.

3.5.3 Discussion of *Vibrio cholerae* infections

There are over 200 different reported serogroups of *V. cholerae* (Chatterjee and Chaudhuri, 2003), but not all are capable of causing cholera. In fact, only CT producing *V. cholerae* strains belonging to the serogroups O1 and O139 are associated with epidemic cholera (Mutreja et al., 2011; Reidl and Klose, 2002). However, other serogroups usually referred as non-O1 and non-O139 strains are occasionally reported to cause systematic infections. Patients with chronic syndromes, such as cirrhosis, hematologic abnormalities, renal dialysis, organ transplants, and immunosuppression appear to be at incased risk of *V. cholerae* non-O1, non-O139 infections (Couzigou et al., 2007; Hou et al., 2011; Phetsouvanh et al., 2008; Stypulkowska-Misiurewicz et al.,

2006; Trubiano et al., 2014). Previous, retrospective studies originating from Taiwan (Hou et al., 2011) and Thailand (Wiwatworapan and Insiripong, 2008) have described patients with cirrhosis and non-toxigenic *Vibrio cholerae* non-O1, non-O139 septicemia. Spontaneous peritonitis has also been observed in patients with *Vibrio cholerae* non-O1, non-O139 septicemia (Ferreira et al., 2012). The more typical manifestations of this infection are ascites, fever, jaundice, diarrhea, skin lesions, and gastrointestinal bleeding (Ferreira et al., 2012; Ko et al., 1998), and we observed the majority of these symptoms in the patients in this report. However, we observed no skin lesions or cellulitis. It has been reported that non-O1, non-O139 *V. cholerae* infections are associated with consumption of, or contact with, raw seafood, which is a similar risk factor for the related *Vibrio* species *V. paraheamolyticus* (Su and Liu, 2007). However, is also noteworthy that neither of the cases described here reported contact with, or consumption of, seafood.

The management of *Vibrio cholerae* non-O1, non-O139 infections differs substantially from epidemic diarrhea. The role of antimicrobials in severe cholera is not as critical as fluid and electrolyte replacement. In contrast, antimicrobials are essential for the management of extra-gastrointestinal *Vibrio* infections, however there are currently no standard guidelines for treating this disseminated infection. Therefore, assessing the antimicrobial susceptibility pattern of the infecting *Vibrio* spp. is paramount for tailoring treatment. Currently *Vibrio cholerae* non-O1, non-O139 from many locations are still reported to be susceptible to beta lactams, fluoroquinolones, trimethoprim-sulfamethoxazole, tetracycline, and chloramphenicol (1,3,8,9,10). It has been suggested that third generation cephalosporins or fluoroquinolones are the most suitable agents for treating *V. cholerae* septicemia. Indeed, ciprofloxacin seems to be associated with a favorable outcome, and we can report here that both patients in this report recovered from the bloodstream infection quickly with a good clinical response after ceftriaxone.

3.6 Outbreak of human Brucellosis in Vietnam

3.6.1 Background

Brucellosis is a collective term for infections caused by small Gram-negative coccobacilli belonging to genus *Brucella*. This genus incorporates the well-described animal pathogens *Brucella melitensis*, *Brucella abortus*, *Brucella ovis*, *Brucella suis*, and *Brucella canis*, which are associated with disease in goats, cattle, sheep, pigs, and dogs, respectively. *Brucella* are facultative intracellular pathogens, and are sequestered by monocytes and macrophages, spreading throughout the body to the liver, spleen, lymph nodes, and bone marrow (de Figueiredo et al., 2015). These pathogens are synonymous with an aggressive disease syndrome in animals causing abortion, stillbirth, and the delivery of weak offspring. The organisms replicate to high concentrations in the affected tissues and are transmitted through contact with the placenta, fetus, fetal fluids, and vaginal discharge. Notably, goats can shed *B. melitensis* in vaginal discharge for up to three months after abortion and organisms can be shed in milk for the lifetime of an infected animal (Tittarelli et al., 2005).

Many *Brucella* species have zoonotic potential and can be transmitted from animals to humans. Brucellosis in humans is typically contracted by contact with infected animals or through the ingestion of animal products prepared from infected animals. In symptomatic cases, disease presentation is highly variable and may arise rapidly or progressively. Classically, brucellosis in humans is a sub-acute, non-specific febrile disease characterized by high temperatures, headaches, malaise, night sweats, and body aches (Jia et al., 2017). Some individuals recover quickly, while others develop more persistent, long-term complications including arthritis, spondylitis, endocarditis, dermatitis, and chronic fatigue, and neurological complications (Jia et al., 2017). The

disease is treated using antimicrobials; however, relapses are common, even after apparent bacteriological cure.

3.6.2 *Brucella* outbreak

From 14th June 2016 to 18th January 2017, ten febrile patients attending HTD had a positive blood culture containing Gram-negative coccobacilli. These organisms were sub-cultured onto sheep chocolate blood agar and subjected to biochemical identification and antimicrobial susceptibility testing. The organisms stained red using a modified cold Ziehl-Neelsen stain, and were identified as *Brucella* spp. on a VITEK2 system (BioMerieux, France). All organisms were susceptible to amikacin, ciprofloxacin, gentamycin, doxycycline, imipenem, rifampicin, and trimethoprim sulphate. Nucleic acid was extracted from organisms and subjected to Bruce-ladder multiplex PCR to identify the infecting species (Lopez-Goni et al., 2008); all produced an identical collection of amplicons indicative of *B. melitensis*. We next performed MLVA-16 (Multiple Locus VNTR Analysis) on the ten *Brucella* isolates (Maquart et al., 2009), which is comprised of three panels, panel 1 (8 minisatellite loci), panel 2A (3 microsatellite loci), and panel 2B (5 microsatellite loci). Panel 1 allows clustering the different *Brucella* species while panels 2A and 2B provide finer resolution characterization. The MLVA profiles of the Vietnamese isolates were compared with a global collection of various *Brucella* species (Figure 3.9). The organisms were all confirmed as *B. melitensis* and produced independent VNTR profiles falling into four subgroups, clustering with organisms originating from Southern Europe, the Middle East, and China.

The presumptive diagnoses of the brucellosis patients prior to bacterial culture and identification were sepsis (patients 1 and 2), non-specific viral infection(3), dengue(4),

tuberculosis(5 and 7), and non-specific inflammatory disease(6); patients 8, 9, and 10 were correctly diagnosed with brucellosis after laboratory diagnosis of the first seven patients. These infections were additionally confirmed using Rose Bengal agglutination with titers in plasma ranging from 1/4 to 1/256. Differential blood counts were largely unremarkable but almost all patients had elevated AST, ALT, and GGT; several patients had elevated CRP and procalcitonin indicating systemic inflammatory response (Table 3.8).



Figure 3. 10 The MLVA genotypes of ten human *Brucella melitensis* cases in southern Vietnam

Circular tree representing the global population structure of the genus *Brucella* using Multiple Locus VNTR Analysis-16 (MLVA-16) analysis. Nine different *Brucella* species (labeled) are shown and associated with main branches; *B. melitensis* forms the majority of the tree. The ten Vietnamese isolates are on black branches within the *B. melitensis* population

Table 3. 9 The clinical characteristics of ten human *Brucella melitensis* cases in southern Vietnam

Patient number	Date of hospital admission	Age (years)	Sex	Potential exposure	Rose Bengal titre	Days in hospital	Days of fever	AST [†] (IU/L)	ALT [†] (IU/L)	GGT [†] (IU/L)	CRP [†] (mg/L)	PCT [†] (ng/mL)
1	06/06/2016	47	Male	Goat keeper/ate goat blood	1/32	16	37	38	35	51	NR	5.04
2	08/26/2016	80	Male	Goat keeper	1/8	21	60	78	64	114	31	2.06
3	08/31/2016	78	Male	Goat keeper	1/64	14	8	69	39	431	NR	0.13
4	09/25/2016	37	Female	Ate undercooked goat meat	1/4	15	6	26	26	NR	NR	NR
5	05/15/2016	40	Male	Goat keeper/had sick animals	1/32	30	14	44	34	235	NR	0.89
	09/26/2016*					15	120	67	61	131	36	0.34
6	10/30/2016	35	Female	Goat keeper	1/16	20	20	295	405	70	64	NR
7	11/23/2016	50	Male	Goat keeper	1/32	8	35	67	61	145	NR	0.12
8	11/16/2016	35	Male	Goat/sheep keeper	1/64	8	30	28	37	167	52	NR
9	12/28/2016	27	Female	Goat keeper	1/16	12	10	515	58	31	7	NR
10	01/18/2017	60	Male	Veterinarian vaccinating goats/sheep	1/256	11	14	55	57	235	NR	NR

* relapse † Normal ranges (results outside normal range in bold, NR; not recorded) AST: 10-40 IU/L, ALT: 5-30 IU/L, GGT: 5-30 IU/L, CRP (C reactive protein): 0-10mg/L, and PCT (Procalcitonin): <0.15 ng/ml

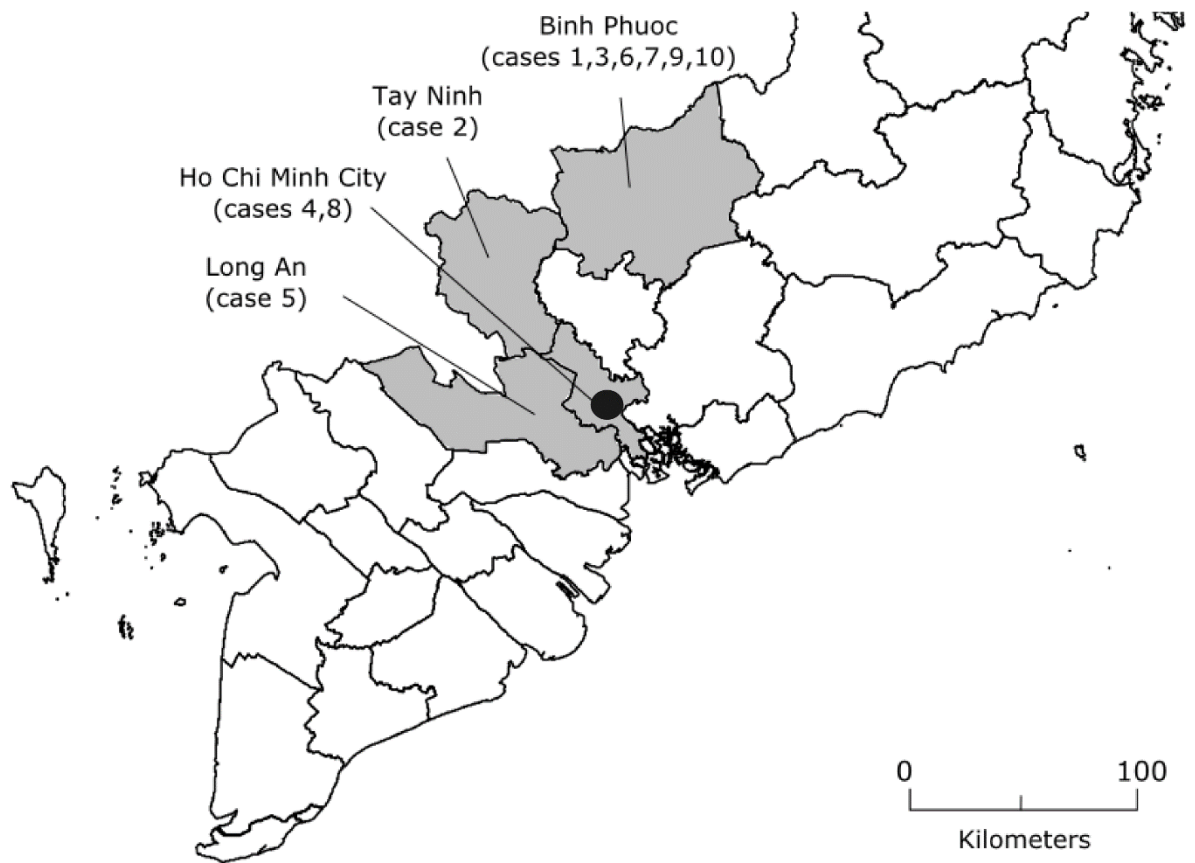


Figure 3. 11 The locations of ten human *Brucella melitensis* cases in southern Vietnam

Map of southern Vietnam showing the locations of provinces where the ten cases of brucellosis originated between June 2016 and January 2017. The solid circle shows the location of the Hospital for Tropical Diseases in Ho Chi Minh City.

On review of the medical histories, all patients had reported exposure to goats prior to the febrile episodes; eight kept goats, two had consumed goat meat, and one was a veterinarian who had been vaccinating goats. The ten cases originated from four provinces, with the primary cases occurring in Binh Phuoc and Tay Ninh near the Cambodian border, and later detection in the south (Ho Chi Minh City and Long An) (Figure 3.10). All patients, apart from patient 5 (ceftriaxone 2g/day for seven days) received doxycycline (200mg/day for six weeks) with gentamycin (240 mg/day for seven days). All patients recovered without relapse with the exception of Patient 5, who was treated with imipenem (2g/day), gentamycin (240mg/day) for seven days and doxycycline (200mg/day) for six weeks. Patient 5 made a complete recovery without additional relapse.

Discussion for *Brucella* outbreak

B. melitensis is a known zoonotic pathogen that can cause an invasive febrile disease in humans exposed to infected animals (Moreno and Moriyon, 2002). Brucellosis infection in humans have been reported from other countries in Southeast Asia such as Thailand, Malaysia (Hartady et al., 2014; Manosuthi et al., 2004). Diagnosing brucellosis in humans is complicated by its non-specific presentation and may not be included in a differential diagnosis. The lack of a confirmatory diagnosis with appropriate antimicrobial therapy can lead to lasting physical effects through chronic infections. Persistent intracellular infection is associated with chronic arthritis, endocarditis and neurobrucellosis. Lewis et al had described *B. melitensis* as a rare cause of prosthetic joint infection from traveller returning from Thailand and other Asia region (Lewis et al., 2016). Here we have reported the first identified cases of human brucellosis caused by *B. melitensis* in Vietnam. The organisms were susceptible to the advocated antimicrobial agents, again highlighting the importance of pathogen isolation and

identification. By performing MLVA genotyping, we found that the organisms fell into four different subgroups, suggesting that these organisms circulate widely in goats in southern Vietnam and were not part of an isolated outbreak. Therefore, we suggest that patients with non-specific febrile disease in Vietnam and comparable locations in Southeast Asia reporting contact with goats, sheep, and cattle receive a blood culture and a Rose-Bengal test. If the diagnosis is strongly suspected, empirical doxycycline can be given.

Vietnam is a hotspot for zoonotic infections and brucellosis is an important disease globally, stemming from the circulation of undiagnosed sick animals coming in contact with humans. About fifty years ago, there was report about *Brucella* infection among human and big cattle in the north of Vietnam which suggests the long-term presence of this bacteria in this country(Quy Nguyen-Phu, 1966). Given the distribution of these cases now and the collective exposure to goats, these findings suggest that these organisms are circulating widely in the goat population, which predicts that human cases may become increasingly common. We recommend that sick animals with a suspicion of brucellosis and their owners be screened in the identified provinces to assess the magnitude of the problem. This type of screening requires an interaction between the Departments of Preventative Medicine and Animal Health. Our work indicates the importance of zoonotic infections in Vietnam and highlights the need for sustained surveillance in human and animal populations.

Chapter 4

The phenotypic and genotypic characteristics of ESBL and AmpC producing organisms associated with bacteraemia in Ho Chi Minh City, Vietnam

4.1 Aims of chapter

Here I aimed to evaluate conventional methods to detect ESBL and AmpC activity in comparison with the molecular method. Therefore, I performed multiplex PCR for several ESBL genes and AmpC genes on clinical isolates from HTD in HCMC with suspected ESBL and AmpC activity. Based on the molecular test results, I will additionally be able to determine the common ESBL and AmpC genes associated with BSI in this major healthcare facility in southern Vietnam. This work has been published at BMC antimicrobial resistance and infection control.

4.2 Introduction

AMR has become an increasing problem in global health. Resistance against antimicrobials used for the empirical therapy of invasive infections is particularly alarming, especially given that the provision of effective antimicrobials rapidly improves the likelihood of a better outcome. To limit the possibility of poor outcomes, broad-spectrum antimicrobials, such as third generation cephalosporins, have become the most commonly empirical drugs to treat non-specific febrile diseases. However, the sustained use of the drugs in hospital settings has meant an increase in resistance to these antimicrobials, which somewhat ironically may lead to a poor outcome. The situation has been internationally recognised and the WHO have identified seven bacteria as the most important cause of AMR infections in hospitals and the community. This list includes *E. coli* and *K. pneumoniae*, which are commonly found to be resistant to third-generation cephalosporins (WHO, 2013).

As previously discussed, resistance to third-generation cephalosporins is mediated by a class of serine hydrolases known as ESBLs, which act by cleaving the β -lactam ring, thereby rendering the drug inactive (Bradford, 2001). With the increasing threat of treatment failure, monitoring ESBL producing organisms in sentinel locations is imperative for surveillance and improved infection control measures.

Routine susceptibility testing is usually capable of detecting the presence of ESBL activity, however false positives can occur. This lack of sensitivity is associated with the plasmid-mediated ampicillinases, AmpC. AmpC genes also confer resistance to many β -lactams and β -lactam/ β -lactamase inhibitor combinations, the latter of which render ESBL producing bacteria susceptible. AmpC genes are generally chromosomally located, however plasmid associated variants of the enzymes have become increasingly recognised. This observation is significant as these ampicillinases can be easily disseminated through horizontal gene transfer (Doi and Paterson, 2007). There are currently no standardised CLSI guidelines for AmpC detection, however several methods have been proposed to aid in the accurate detection of AmpC β -lactamases (Black et al., 2005; Gupta et al., 2014; George A. Jacoby, 2009). These phenotypic tests primarily use a cephalosporin with a β -lactamase inhibitor (e.g. clavulanate) or non- β -lactamase inhibitors (e.g. boronic acid) and can be combined with molecular detection of the AmpC β -lactamases using multiplex PCR (Pérez-Pérez and Hanson, 2002). However, due to overexpression of AmpC genes, bacteria carrying an ESBL gene may test negative for ESBL production and subsequently interpretations of phenotypic AmpC methods (Song et al., 2005; Steward et al., 2001; Thomson, 2001) which can be catastrophic for treatment outcomes. The accurate testing and interpretation of AmpC and ESBL activity is vital for healthcare professionals to provide effective and appropriate treatment management.

Here, I aimed to assess the distribution of AmpC and ESBL genes in organisms associated with bloodstream infections between January 1st 2011 and December 31st 2013 at HTD in HCMC, Vietnam. My data highlights the need for routine AmpC and ESBL gene surveillance in hospitals in low-middle income countries to ensure effective infection control measures and report on the incidence of drug resistant bacteraemia.

4.3 Results

4.3.1 Gram-negative organisms with reduced susceptibility against third-generation cephalosporins isolated from bloodstream infections at the Hospital for Tropical Diseases

Between January 2011 and December 2013, 1,690 non-contaminant bacteria were isolated from the blood of febrile patients attending HTD in HCMC. The overall isolation rate of from blood during this period was 5.6% (1,690/30,185); 1,017 of these were Gram-negative (excluding *Pseudomonas* spp. and *Acinetobacter* spp.). Ceftriaxone (or an alternative parenteral third-generation cephalosporin) was (and remains) the empirical therapy for suspected bacteraemia in this healthcare facility. Therefore, all Gram-negative organisms isolated from blood were routinely screening for susceptibility against third-generation cephalosporins. In total, nearly a third (304/1,017) were found to exhibit reduced susceptibility against third-generation cephalosporins, with >90% (n=280) of these exhibiting resistance to ceftriaxone and nearly 3% (n=8) being intermediate according to current CLSI susceptibility breakpoints (Clinical and Laboratory Standards Institute, 2010).

The 280 non-duplicate organisms with reduced susceptibility to third-generation cephalosporins were subjected to phenotypic screening using the double disk diffusion method for ESBL activity, and to detect a zone of reduced inhibition between the third-

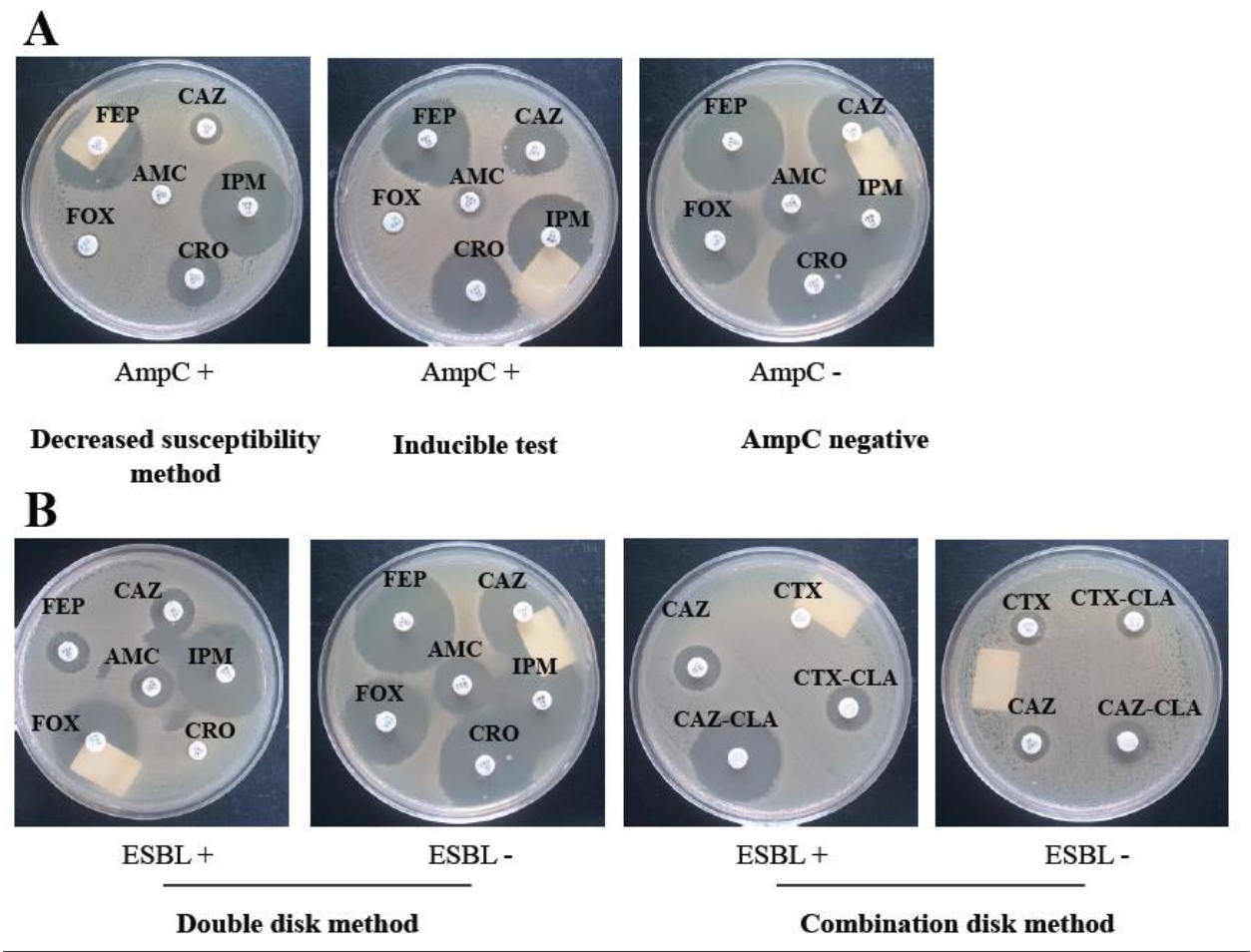
generation cephalosporin and imipenem, indicative of AmpC activity (Figure 4.1). In total 172/1,017 (16.9%) isolates were phenotypically ESBL positive and 63/1,017 (6.2%) were phenotypically AmpC positive, 5/1,017 (0.5%) exhibited phenotypic evidence of both ESBL and AmpC activity. *E. coli* was the most common bacterial species exhibiting ESBL activity, accounting for >90% (162/177) of this group of ceftriaxone resistant organisms (Figure 4.2). Approximately 40% (25/63) of the AmpC producing organisms were *Aeromonas* spp.; *E. coli* was the second most abundant species amongst the AmpC producers with 21/6 (>30%) identified. The remaining organisms exhibiting AmpC activity belonged to a range of species, including *Enterobacter* spp. (8/63), *K. pneumoniae* (7/63), and *Morganella morganii* (2/63).

4.3.2 Molecular analysis of ESBL and AmpC genes conferring resistance to third-generation cephalosporins

PCR amplification was performed on nucleic acid extracted from the 177 ESBL (172 ESBL and 5 producing both ESBL and AmpC) exhibiting phenotypic ESBL activity to identify four ESBL gene groups (*bla*_{TEM}, *bla*_{SHV}, *bla*_{OXA}, and *bla*_{CTX-M}). 67/177 (38%) isolates had only one ESBL gene in which *bla*_{CTX-M} was the most prevalent in 63/177 (36%). There were 106/177 (60%) isolates had combination of two or more ESBL genes, in which the *bla*_{CTX-M} and *bla*_{TEM} complex was the most common combination (67/177; 38%) (Table 4.5). These PCR amplifications demonstrated that *bla*_{CTX-M} was the most prevalent ESBL gene family in these organisms, testing positive in 168/177 (95 %) nucleic acid extractions. Subsequent sequencing of the *bla*_{CTX-M} PCR amplicons revealed that *bla*_{CTX-M-15} (n=84) was the most common ESBL gene subtype. Other ESBL gene subtypes detected included *bla*_{CTX-14} (n=40), *bla*_{CTX-24} (n=6), *bla*_{CTX-27} (n=48), and *bla*_{CTX-55} (n=12) (Table 4.1). Of the five isolates exhibiting both an ESBL and an AmpC phenotype, only two had genetic determinants that may induce an AmpC

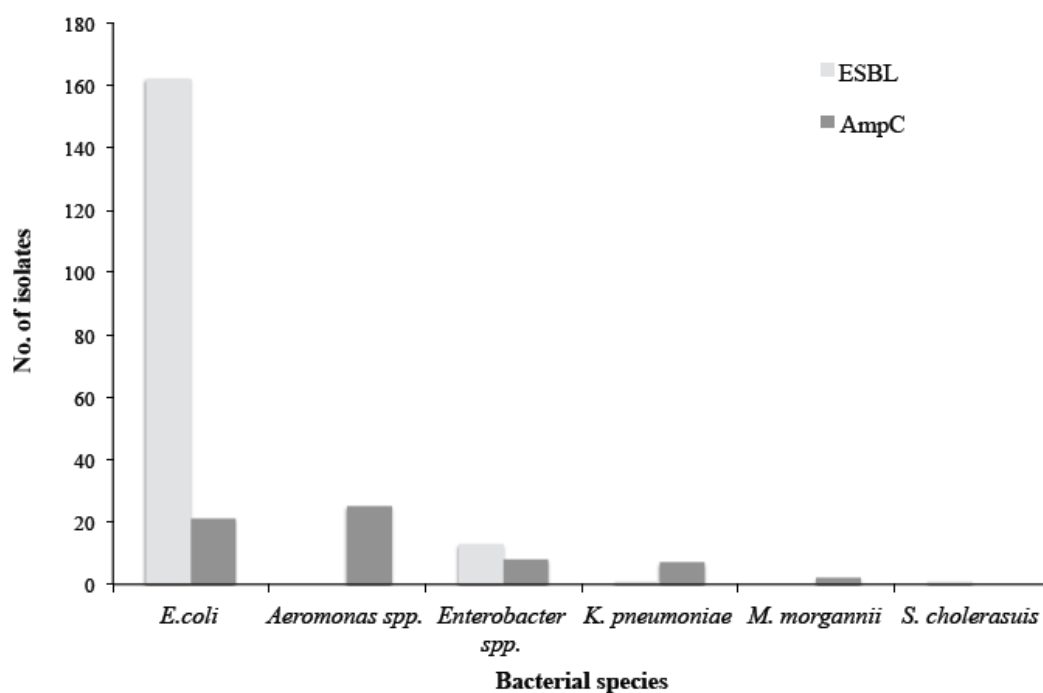
phenotype, namely, *bla*_{CIT} (CMY-42) and *bla*_{EBC}. Two *bla*_{CTX-M} variants (*bla*_{CTX-M-15} and *bla*_{CTX-M-27}) were identified in these organisms as the genes most likely to confer resistance against third generation cephalosporins (Table 4.2).

Figure 4. 1 Representative results of the double disk diffusion test (A) for ESBL production and the AmpC disk test (B)



Abbreviations. AMC: amoxicillin, CTX: cefotaxime, CAZ: ceftazidime, CRO: ceftriaxone, CLA: clavulanate, FEP: cefepime, FOX: ceftazidime, IPM: imipenem.

Figure 4. 2 The distribution of ESBL or AmpC producing bacteria isolated during the period of 2011-2013



Bar chart indicates the number and type of ESBL or AmpC producing bacteria isolated.

PCR amplification of nucleic acid extracted from the 63 organisms with indicative AmpC activity only found that *bla_{CTT}* was the most common gene associated with this phenotype and resistance to third generation cephalosporins; this variant was detected in 18/63 (29%) of the isolates (Table 4.3). Sequencing of the AmpC amplicons determined that approximately half (11/18; 61%) of the isolates harboured the *bla_{CMY-2}* subtype; the remainder (7/18; 39%) carried the *bla_{CMY-42}* gene. Notably, more than half (34/63; 54%) of the organisms conferring an AmpC phenotype did not generate a PCR amplicon for any of the six AmpC genetic markers that were screened. The majority of the organisms exhibiting AmpC activity but not generating any detectable PCR amplifications were *Aeromonas* spp. The remainder of the AmpC producing organisms harboured either *bla_{DHA}* (9/64; 14%) or *bla_{EBC}* (2/64; 3%) genes. Almost half (27/63; 43%) of the AmpC producing isolates also generated a PCR amplicon for an ESBL gene. The most commonly detected ESBL gene in these phenotypic AmpC organisms was the *bla_{TEM}* gene, found in 17/27 (30%) of these isolates (Table 4.4). One *E. coli* isolate harboured three different β -lactamase genes, *bla_{CTX-M}*, *bla_{TEM}*, and *bla_{OXA}*.

Table 4. 1 The distribution of β -lactamase genes identified in 177 ESBL producing bacteria

Gene	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Enterobacter</i> <i>cloacae</i>	<i>Salmonella</i> <i>cholerasuis</i>	Total*
<i>bla</i> _{CTX-M}	157	9	1	1	168 (95%)
<i>bla</i> _{TEM}	70	7	1	1	79 (45%)
<i>bla</i> _{OXA}	33	2	1	-	36 (20%)
<i>bla</i> _{SHV}	1	4	-	-	5 (3%)
Not detected	3	1	-	-	4 (2%)

* The proportion of bacterial species harbouring a particular ESBL gene is expressed as a percentage of the total bacteria possessing an ESBL phenotype (n=177).

Table 4. 2 The Genetic determinants associated with the five ESBL and AmpC producers

Strain	Organism	ESBL gene	AmpC gene
E063	<i>E. coli</i>	<i>bla</i> _{CTX-M-27}	<i>bla</i> _{CTT} *
E080	<i>E. cloacae</i>	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM}	<i>bla</i> _{EBC} [#]
E081	<i>E. coli</i>	<i>bla</i> _{CTX-M-27}	-
E113	<i>K. pneumoniae</i>	<i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA} , <i>bla</i> _{TEM}	-
E126	<i>E. coli</i>	<i>bla</i> _{CTX-M-27}	-

*CMY-42 was the *bla*_{CTT} variant identified by DNA sequencing. [#] The *bla*_{EBC} variants includes (MIR-1 and ACT-1)

Table 4. 3 The distribution of β -lactamase genes within the AmpC producing bacteria

Gene	<i>Aeromonas</i> spp.	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Enterobacter</i> spp.	<i>M. morganii</i>	Total*
<i>bla</i> _{CIT}	-	18	-	1	-	18 (29%)
<i>Bl</i> _{DHA}	-	1	6	-	2	9 (14%)
<i>Bla</i> _{EBC}	-	-	-	2	-	2 (3%)
Not detected	25	3	1	5	-	34 (54%)

*The proportion of bacterial species harbouring a particular AmpC gene is expressed as a percentage of the total bacteria possessing an AmpC phenotype (n=63).

4.3.3 Hospital and community-acquired third-generation cephalosporin resistant infections

E.coli and *Aeromonas* spp. were the most common bacterial species identified as being either ESBL or AmpC producing, respectively. Ninety-seven percent (157/162) of the *E. coli* isolates with an ESBL phenotype harboured a *bla*_{CTX-M} gene. Of these, 135 were associated with a CAI and 22 were associated with a HAI. Hypothesising that organisms associated with HAI would have a broader range of AMR, I additionally aimed to compare the antimicrobial susceptibility profiles of organisms associated with CAIs and HAIs. However, after investigating resistance to a range of different antimicrobial classes in the ESBL producing organisms, I found no significant difference between the antimicrobial susceptibility profiles of organisms associated with CAI or HAI ($p>0.05$; student t-test) (Figure 4.3). I additionally compared the antimicrobial susceptibility phenotypes between the *E. coli* associated with CAI and HAI for the isolates that were phenotypically AmpC positive; there was no significant difference between these groups ($p>0.05$; student t-test) (Figure 4.4). Lastly, I investigated the clinical source of the AmpC producing *Aeromonas* spp. in CAI and HAI. I found that the AmpC expressing *Aeromonas* spp. were significantly associated ($p<0.001$; student t-test) with liver cirrhosis/hepatitis in both CAI and HAI (Figure 4.5).

Table 4. 4 The distribution of the 68 AmpC producing bacteria harbouring an additional ESBL gene

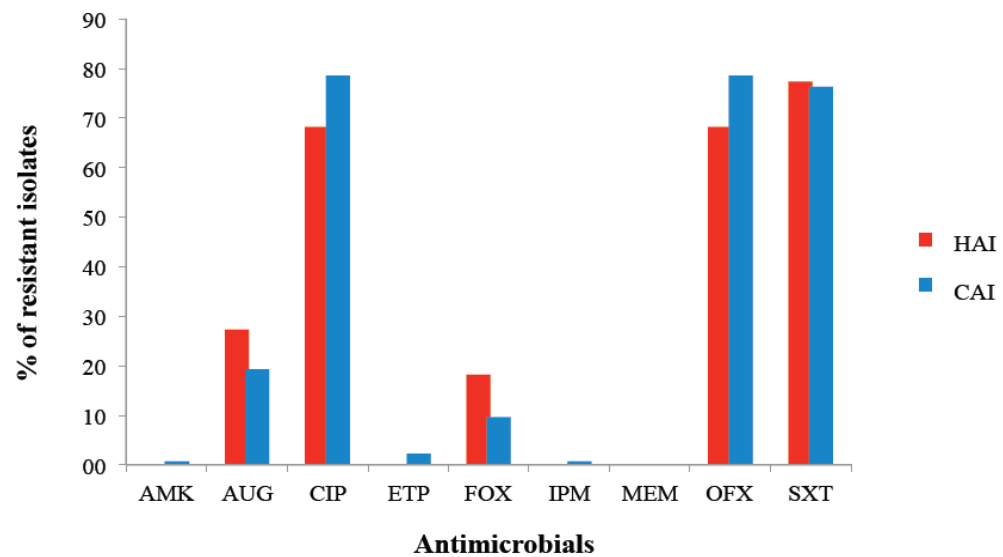
Gene	<i>Aeromonas</i> spp.	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Enterobacter</i> spp.	<i>M. morganii</i>	Total*
<i>bla</i> _{CTX-M}		3				3 (4%)
<i>bla</i> _{TEM}	-	15	-	1	1	17 (25%)
<i>bla</i> _{SHV}	-	-	4	1	-	5 (7%)
<i>bla</i> _{OXA}	-	2	-	-	-	2 (3%)
<i>bla</i> _{OXA} & <i>bla</i> _{TEM}	-	1	1	-	-	2 (3%)
<i>bla</i> _{OXA} , <i>bla</i> _{TEM} & <i>bla</i> _{CTX-M}	-	1	1	1	-	3 (4%)
Not detected**	25	2	2	6	1	36 (53%)

*The proportion of all bacterial species with AmpC phenotype (n=68) gene is expressed as a percentage of the total bacteria possessing harbouring an additional ESBL gene. **These samples had an ESBLs phenotype but no detectable ESBL gene

Table 4. 5 The distribution of several ESBL genes in 177 ESBL producing bacteria

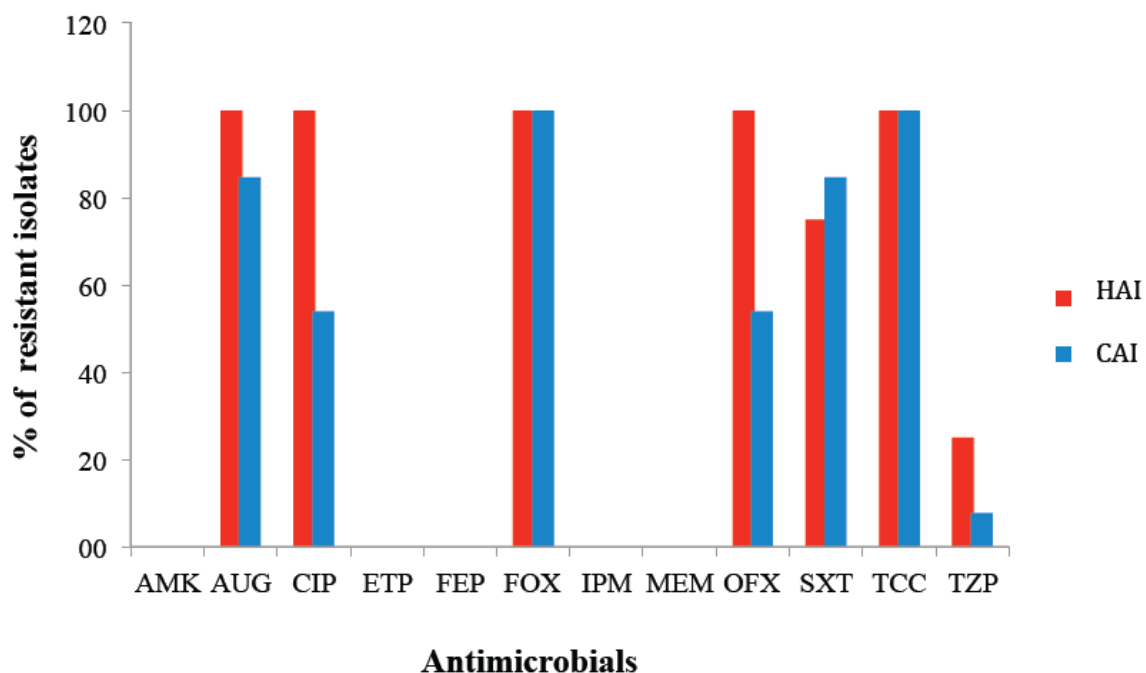
Genes		Number of isolates (%)	
No ESBL gene		4 (2.3%)	4 (2.3%)
Single genes	<i>bla</i> _{CTX-M}	63 (35.6%)	67 (37.8%)
	<i>bla</i> _{TEM}	2 (1.1%)	
	<i>bla</i> _{SHV}	2 (1.1%)	
	<i>bla</i> _{OXA}	0 (0%)	
Multiple-genes	<i>bla</i>_{CTX-M} and <i>bla</i>_{TEM}	67 (37.9%)	106 (59.9%)
	<i>bla</i> _{CTX-M} and <i>bla</i> _{OXA}	27 (15.3%)	
	<i>bla</i> _{CTX-M} and <i>bla</i> _{SHV}	2 (1.1%)	
	<i>bla</i> _{TEM} and <i>bla</i> _{OXA}	1 (0.6%)	
	<i>bla</i> _{CTX-M} , <i>bla</i> _{TEM} and <i>bla</i> _{OXA}	8 (4.5%)	
	<i>bla</i> _{CTX-M} , <i>bla</i> _{TEM} and <i>bla</i> _{SHV}	1 (0.6%)	

Figure 4. 3 A comparison of antimicrobial susceptibility profiles between CAI and HAI of the 177 ESBL producing *E. coli*



The AMR phenotype of the ESBL producing *E. coli* (n=157) from HAI (n=135) or CAI (n=22) was scored for resistance to each drug and this was expressed as a percentage relative to the number of organisms in each group (CAI or HAI). No significant differences ($p>0.05$) were found in antimicrobial resistance phenotype between CAI and HAI.

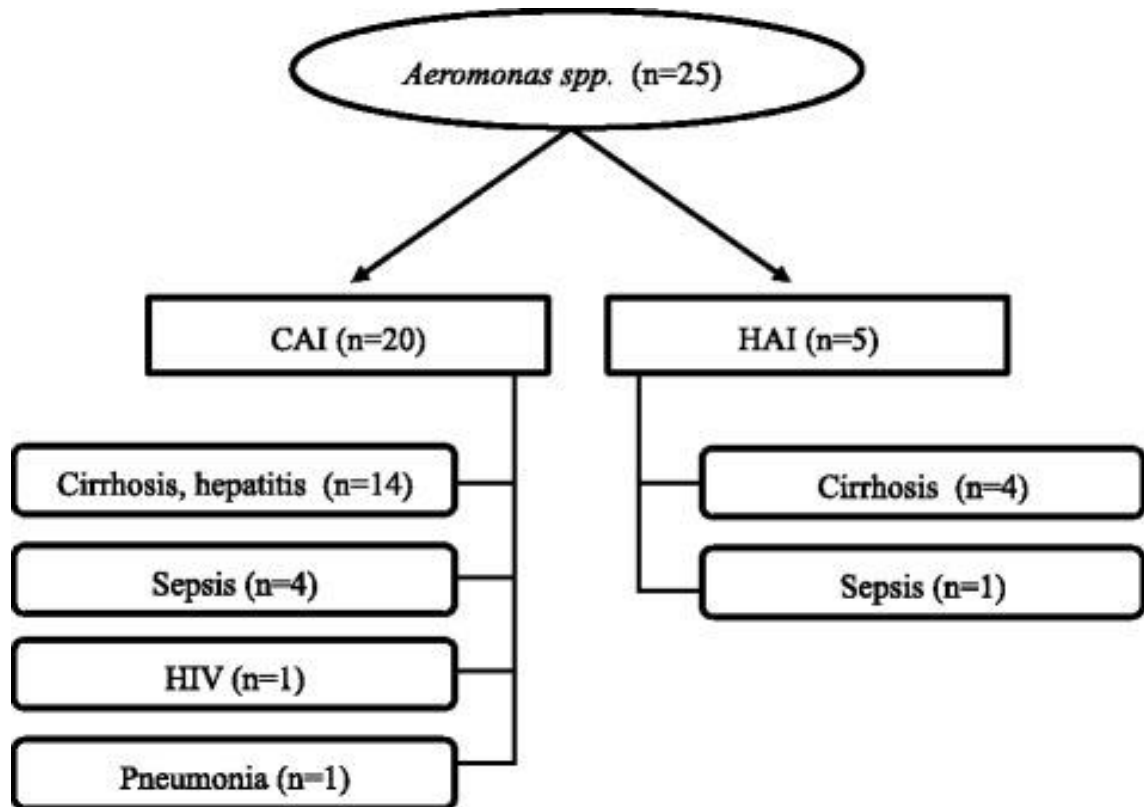
Figure 4. 4 Comparison of antimicrobial susceptibility profiles between CAI and HAI of 63 AmpC producing *E. coli*



The AMR phenotype of the AmpC producing *E. coli* (n=17) from HAI (n=4) or CAI (n=13) was scored for resistance to each drug and this was expressed as a percentage relative to the number of organisms in each group (CAI or HAI). No significant differences ($p>0.05$) were found in antimicrobial resistance phenotype between CAI and HAI.

AMK: amikacin, AUG: amoxicillin-clavulanate, CIP: ciprofloxacin, ETP: ertapenem, FOX; ceftiofur, IMP: imipenem, MEM: meropenem, OFX: ofloxacin, SXT: trimethoprim-sulfamethoxazole, TCC: ticarcillin-clavulanate, TZP: piperacillin-tazobactam.

Figure 4. 5 The distribution of bacteremia infections where *Aeromonas* spp. was isolated from community (CAI) and hospital (HAI) patients



From 63 AmpC producing bacteria isolates, 25 were found to be *Aeromonas* spp. Indicated below the CAI/HAI designation, are the infections the patients presented at the point of sample collection. We found cirrhosis/hepatitis to be significantly associated ($p<0.001$) infection by *Aeromonas* spp.

4.4 Discussion

With the ever-increasing reports of ESBL producing Gram-negative bacteria in clinical settings, identifying these organisms is imperative for monitoring and the provision of efficacious treatments. Although antimicrobial susceptibility profiling is still the most commonly used method, interpretation of AmpC production data can sometimes prove problematic and may lead to under-reporting or misdiagnosis as an ESBL phenotype (Hanson, 2003). This scenario could potentially lead to treatment failure due to 3rd cephalosporin. Here, I aimed to investigate the prevalence and diversity of ESBL and AmpC producing isolates from HTD from organisms isolated from bloodstream infections only over a three-year period.

CTX-M type ESBLs are the most prevalent enzymes that are reported, with over 170 different subtypes assigned (Lahey Clinic, 2016; Pitout and Laupland, 2008). This investigation identified that these enzymes were largely associated with *E. coli* isolates originating from the community. This was not unexpected, as a previous study from the same region found that healthy individuals had a high carriage rate of ESBL producing *Enterobacteriaceae* in their gastrointestinal microbiota (Vien et al., 2009). Furthermore, I found no statistical significance in overall AMR carriage between CAI and HAI in *E. coli* harbouring an ESBL or AmpC phenotype. This observation is important and suggests equilibrium in the circulating AMR organisms in the community and clinical setting with the potential to cause disease in this location. I additionally identified that approximately half of the AmpC producing organisms additionally harbour an ESBL gene. The presence of a co-producing ESBL may affect AmpC susceptibility interpretation (Hanson, 2003; Song et al., 2005; Thomson et al., 2004), therefore the treatment of infections caused by suspected AmpC producing organisms with cefepime requires additionally microbiological assessment .

No plasmid associated resistance genes were detected for the most prevalent AmpC producing organism identified in this study, *Aeromonas* spp. I therefore speculate that the AmpC phenotype in these organisms was mediated by a chromosomally located AmpC gene (George A. Jacoby, 2009; Philippon et al., 2002). Notably, when assessing the clinical presentations associated with the AmpC producing *Aeromonas* spp., these organisms were found to be associated with cirrhosis/hepatitis. *Aeromonas* spp. are commonly identified as the agent of bacteraemia in cirrhosis patients, and commonly has a higher mortality rate than infections caused by alternative pathogens (Lau et al., 2000; Llopis et al., 2004). The high prevalence of *Aeromonas* spp. mediated cirrhotic infections suggests the spread of this bacterial organism within immunocompromised chronic liver patients in HCMC, a potential association that requires longitudinal surveillance.

I observed a high prevalence of AmpC and ESBL expressing organisms associated with both CAI and HAI in HCMC. AmpC producing *Aeromonas* spp. appear to potentially be associated with bacteraemia in cirrhosis/hepatitis patients. With febrile disease presentations, it is routine clinical practice to prescribe a broad-spectrum antimicrobial, generally a third-generation cephalosporin, to increase the likelihood of a better clinical outcome (Kollef, 2003, 2008). However, my data suggest this approach may not be optimum and the use of third-generation cephalosporins in monotherapy should be assessed carefully when there is a clinical suspicion of bacteraemia, irrespective of whether this is a suspected CAI or HAI.

Chapter 5

Invasive non-typhoidal *Salmonella* infections in Asia: clinical observations, disease outcome and dominant serovars from an infectious disease hospital in Vietnam

5.1 Aims of chapter

I hypothesise that the epidemiology of invasive NTS infections is ecologically different to that of sub-Saharan Africa, and I predict due to improved access to hospital care, antiretroviral drugs and nutrition that mortality is low and limited to a few specific risk factors such as liver damage. To address this hypothesis I conducted a retrospective analysis on all NTS patients hospitalised at HTD in HCMC over a four-year period. Data was collected from records, entered into a custom designed database and stratified by HIV status, age, sex, nutrition, treatment, hospital stay, complications, gastrointestinal complaints, and outcome. Data was described and then analysed to assess risk factors such as poor outcome, such as prolonged hospital stay, relapse, complications, co-morbidities and death. This was the first such study of its type originating from outside sub-Saharan Africa and was published in PLoS Neglected Tropical Diseases in 2016.

5.2 Introduction

Salmonella bacterial infections in humans can cause a range of syndromes. A subset of the >2,500 described serovars of *Salmonella* subspecies I can cause typhoidal illness, including *S. Typhi* and the various *S. Paratyphi* pathovars (Popoff et al., 2004). However, the great majority of *Salmonella* infections in humans do not generally cause systemic disease and are referred to as NTS. NTS organisms include *S. Typhimurium*, *S. Dublin* and *S. Enteritidis*, which are characterized by their wide host range and their

ability to induce self-limiting diarrhea in humans (Crump et al., 2015). However, in addition to the common diarrheal clinical syndrome induced by NTS organisms in humans, invasive (bloodstream) NTS (iNTS) disease also occurs in specific populations (Crump and Heyderman, 2015; Feasey et al., 2012). iNTS disease, which is most commonly caused by the *Salmonella* serovars Typhimurium and Enteritidis (Feasey et al., 2015; Kariuki and Onsare, 2015), is associated with an aggressive systemic infection that can resemble typhoid fever (Crump et al., 2015; J. A. Crump et al., 2011; Crump and Heyderman, 2015). In sub-Saharan Africa, the disease has a high mortality rate (20-25%) and infection is most common in children with malaria, malnourished children and HIV-infected adults (Feasey et al., 2012). There are an estimated 1.9 million cases of iNTS disease in Africa annually, with incidences rate of 227 per 100,000 population overall (Ao et al., 2015) and 175-388 and 2,000-7,500 per 100,000 population in children 3-5 years of age and HIV-infected individuals, respectively (Bassat et al., 2009; Berkley et al., 2005; Enwere et al., 2006; Feasey et al., 2012; Gordon et al., 2008; Oosterhout et al., 2005; Reddy et al., 2011).

NTS are a common cause of diarrhea in Asia, and previous work has shown that NTS are responsible for approximately 4% of pediatric hospitalized diarrhea in HCMC (Thompson et al., 2015). In a retrospective study of blood cultures conducted between 1994 and 2008 at the HTD in HCMC it was observed that *S. Typhi* was the predominant cause of culture positive bacteremia (66%) until 2002 (Nga et al., 2012). After this period there was significant annual decline in the isolation rate of *S. Typhi* and a concurrent increase in organisms associated with the HIV epidemic, including NTS. The isolation rate of NTS increased from 1% (n=47) of total bacteremia cases between 1994-2001 to 4% (n=146) of cases from 2002-2008. Whilst the increase in burden was modest in comparison to sub-Saharan Africa these data support a longitudinal shift in the etiology of bloodstream infections in southern Vietnam.

There is a paucity of data regarding iNTS infections from Asia, with limited reports from Taiwan (Chen et al., 2012), India (Menezes et al., 2010), Thailand (Hendriksen et al., 2012; Kiratisin, 2008) and the aforementioned study in Vietnam (Nga et al., 2012). It is apparent that the burden of iNTS in sub-Saharan Africa is not mirrored in Asia. However, iNTS disease is present in Asia but there are no or few data regarding clinical symptoms, disease outcome, patient demographics or the infecting serovars. By accessing available clinical data and bacterial isolates I sought to retrospectively investigate the clinical and microbiological manifestations of iNTS in a major infectious disease hospital in southern Vietnam.

5.3 Results

5.3.1 The demographic and laboratory features of invasive non-typhoidal *Salmonella* infections

Between January 2008 and June 2013 there were 142 culture confirmed bloodstream infections caused by an NTS bacterium at HTD. Hospital records were obtainable for 102/142 (72%) iNTS cases. The median patient age was 33 years (IQR: 28 to 41 years) (Table 5.1). Eight of the 102 (8%) iNTS cases were children (<16 years) of which five (5% of total) were infants (<12 months). The majority of patients (61/102; 60%) were from HCMC, with the remainder residing in the surrounding provinces. The median duration of illness (including fever and other symptoms) prior to hospital admission was 13 days (IQR 1-60 days). Patients were more commonly male (72/102; 71%) and three quarters (71/94; 76%) of adults (>16 years) reported that they were unemployed upon admission. A third (31/102; 33%) of cases reported a history of intravenous drug use, which was more common in men (26/65, 40%) than women (5/30, 17%) ($p=0.019$, Fisher's exact test).

HIV testing was performed for all patients diagnosed with iNTS infections; CD4 counts were not routinely measured. Seventy-two (71%) of the iNTS cases were HIV-infected: 71 adults (76% of 94 adults) and one infant (13% of all 8 children). Only 16/72 (22%) of the adult HIV-infected iNTS patients were on active antiretroviral therapy (ART) prior to this episode of bacteremia, and 6/72 (8%) of the HIV-infected iNTS patients were taking trimethoprim-sulfamethoxazole for *Pneumocystis jiroveci* pneumonia prophylaxis on admission. A history of long-term steroid use was reported in 4/30 (13%) of the iNTS cases testing negative for HIV infection.

Table 5. 1 The clinical characteristics of invasive non-typhoidal *Salmonella* disease stratified by HIV status and outcome

Characteristic	Total n=102	HIV status		Fatal ^a	
		Infected n=72	Uninfected n=30	Yes n=26	No n=66
Male sex	72 (70.6)	54 (75.0)	18 (60.0)	18 (69.2)	44 (66.7)
Age	33 (28-41)	31.5 (28-37)	44 (25-69)	33.5 (29-37)	33 (27-44)
Reported Injected drug user	31/95 (32.6)	31/65 (47.7)	0 (0)	12/25 (48)	15/60 (25)
Immunosuppression					
HIV infection	72 (70.6)	72 (100)	0 (0)	24 (92.3)	39 (59.1)
On ART (HIV infected)	16/72 (22.2)	16/72 (22.2)	0/20 (0)	8/24 (33.3)	7/39 (17.9)
Hepatitis	27 (27.0)	20/70 (28.6)	7 (23.3)	8/25 (32.0)	17 (25.8)
Symptoms and signs on admission					
Abdominal pain	23 (22.5)	14 (19.4)	9 (30.0)	5 (19.2)	17 (25.8)
Ascites	8 (7.8)	8 (11.1)	0 (0)	2 (7.7)	4 (6.1)
Cough	44 (43.1)	35 (48.6)	9 (30.0)	14 (53.8)	26 (39.4)
Diarrhea	42 (41.2)	28 (38.9)	14 (46.7)	11 (42.3)	26 (39.4)
Fever	76/97 (78.4)	49/69 (71)	27/28 (96.4)	15/24 (62.5)	54/63 (85.7)
Hepatomegaly	25 (24.5)	21 (29.2)	4 (13.3)	4 (15.4)	18 (27.3)
Oral candidiasis	36 (35.3)	35 (48.6)	1 (3.3)	11 (42.3)	21 (31.8)
Pallor	56/101 (55.4)	38/71 (53.5)	18 (60.0)	15 (57.7)	35 (53.0)
Pneumonia	72 (70.6)	56 (77.8)	16 (53.3)	19 (73.1)	45 (68.2)
Septic Shock	6 (5.9)	4 (5.6)	2 (6.7)	4 (15.4)	2 (3.0)
Splenomegaly	16 (15.7)	15 (20.8)	1 (3.3)	3 (11.5)	10 (15.2)
Tachypnea	29 (28.4)	19 (26.4)	10 (33.3)	13 (50.0)	15 (22.7)
Throat lesions	45 (44.1)	40 (55.6)	5 (16.7)	13 (50.0)	26 (39.4)
Coinfections	16 (15.7)	16 (22.2)	2 (6.7)	6 (23.1)	10 (15.2)
<i>T. marneffei</i>	9 (8.8)	9 (12.5)	0 (0)	2 (7.7)	7 (10.6)
Other ^b	7 (6.9)	7 (9.7)	0 (0)	4 (15.4)	3 (4.5)
Serovar					
<i>S. Enteritidis</i>	43/89 (48.3)	30/63 (47.6)	13/26 (50.0)	12/22 (54.5)	26/57 (45.6)
<i>S. Typhimurium</i>	23/89 (25.8)	22/63 (34.9)	1/26 (3.8)	7/22 (31.8)	11/57 (19.3)
<i>S. Choleraesuis</i>	11/89 (12.4)	9/63 (14.3)	2/26 (7.7)	1/22 (4.5)	10/57 (17.5)
Other	12/89 (13.5)	2/63 (3.2)	10/26 (38.5)	2/22 (9.1)	10/55 (18.2)

a) 10 patients missing outcome status; b) Includes *Cryptococcus neoformans*, *Streptococcus pneumoniae* and *Escherichia coli* isolates cultured from either blood or cerebrospinal fluid

Table 5.1 describes the clinical characteristics of the patients. The most common clinical features were fever (76/97, 78%) ($\geq 38.0^{\circ}\text{C}$) and pallor (56/101, 56%). Almost half of the cases (45/102) had oropharyngeal lesions, including ulcers and candidiasis; these symptoms were chiefly restricted to the HIV-infected group. Notably, gastrointestinal symptoms such as diarrhea (42/102, 41%) and abdominal pain (23/102, 23%), which are synonymous with the archetypal, non-invasive manifestation of NTS infection, were uncommon. However, comorbidities such as hepatitis (induced by hepatitis B, C or alcohol abuse) and pneumonia (caused by PCP or *Mycobacterium tuberculosis*) were recorded in 27% (27/102) and 71% (72/102) of patients, respectively. Furthermore, 16/102 (16%) patients had an additional pathogen identified in their bloodstream (BS) or cerebrospinal fluid (CSF): 9 *Talaromyces marneffeii* (BS), 4 *Cryptococcus neoformans* (CSF), 2 *Escherichia coli* (BS) and 1 *Streptococcus pneumoniae* (BS). None of these additional BS or CSF infections were identified in children. Septic shock was diagnosed in 6/102 (6%) cases; hypovolemic shock (due to fluid loss) was diagnosed in 2/102 (2%); a secondary infection was identified in only 1/6 (17%) patient with septic shock. Furthermore, 2/8 (25%) of the paediatric patients were diagnosed with hand-foot and mouth disease prior to the isolation of an NTS organism from the blood.

Table 5. 2 Laboratory results of invasive non-typhoidal *Salmonella* disease stratified by outcome

Laboratory test	Normal range	Total cases (n=102) Median (IQR)	Fatal (n=26) Median (IQR)	Nonfatal (n=66) Median (IQR)	<i>p</i> value ^a
White blood cells (10 ³ cells/μl)	3.7-11.7	5.1 (3.1-10.8)	4.5 (2.9-6.5)	6.9 (3.0-11.1)	0.210
Neutrophils (%)	39.6-78.4	82 (66.1-87.5)	84.7 (74.05-88)	77.6 (63.8-85.3)	0.479
Lymphocytes (%)	14.1-52.8	11.2 (4.8-19.2)	8.05 (4.8-18.4)	12.4 (5.6-20.3)	0.261
Platelets (10 ⁹ cells/μl)	172-440	142 (58-258)	90 (59-153)	178 (64-269)	0.042
Hemoglobin (g/dL)	12.0-17.2	10.2 (7.9-12)	8.1 (6.95-10.1)	10.8 (8.9-12.5)	0.001
Hematocrit (%)	34.8-50.9	30.1 (23.6-36.1)	25.2 (20.8-30.4)	31.9 (26.8-37.6)	0.002
AST (U/L)	5-30	100.4 (52.4-189)	109 (52.4-320)	90.5 (48-133)	0.094
ALT (U/L)	5-30	47 (26-93)	36 (22-74)	47 (27.5-84.4)	0.547
Creatinine (μmol/L)	80-130	68 (57-107)	74.5 (49.5-172)	68 (55-87)	0.303
Sodium (mmol/L)	135-145	130 (126-134)	130.9 (125-135.5)	130 (127-134)	0.900
Potassium (mmol/L)	3.5-5.0	3.7 (3.3-4.5)	4.3 (3.6-4.8)	3.6 (3.1-3.9)	0.003

a) Derived using Kruskal-Wallis test; normal values derived from minimum and maximum adult values (>18 years) listed in (Farinde, 2016; Virginia Commonwealth University, 2010)

5.3.2 Outcome of invasive non-typhoidal *Salmonella* infections

Overall 66/102 (65%) patients improved or recovered before hospital discharge; four (4%) died in hospital and 22 (22%) were discharged to die at home; the remaining 10 patients had an outcome that was non-assessable (five left against medical advice, two were unchanged and three transferred hospitals). One child (1/8, 12%), who was HIV-uninfected, died. The overall mortality rate was 26/92 (28%), of which 24 (92%) were HIV-infected. A total of 23% (6/26) of fatal cases had a secondary infection in bloodstream or central-spinal fluid. The median time to death in hospital was one day (IQR: 1-2 days) while median length of hospital stay for patients not discharged to die at home was 10 days (IQR: 3-15 days).

Hematology parameters for the 102 patients stratified by outcome are shown in Table 5.2. Notably, total white cell count was generally non elevated (median 5.1 (IQR: 3.1-10.8) $\times 10^3$ cells/ μ l) but characterized by a high proportion of neutrophils: 82% (IQR: 66.1-87.5). The platelet count was lower in fatal cases than nonfatal cases but this was not statistically significant. Fatal cases were significantly more likely to have higher potassium, lower haemoglobin, and lower hematocrite levels (Table 5.2). Additionally, I performed univariable and multivariable logistic regression analyses to assess the clinical and laboratory variables that were associated with death (Table 5.3). Though HIV positivity, age and infecting serovar were associated with death in the univariable analysis, after controlling for confounding only HIV positivity remained independently associated with an increased risk of fatality (Table 5.3).

5.3.3 The treatment of invasive non-typhoidal *Salmonella* infections

The vast majority of iNTS patients received an antimicrobial (100/102; 98%) (Table 5.4). The most commonly used antimicrobial was ceftriaxone; 89/100 (89%) patients received this drug in mono or combination-therapy. A fluoroquinolone (levofloxacin, ciprofloxacin or ofloxacin) was used in 22/100 (22%) of cases, again either used in monotherapy or in combination with ceftriaxone (Table 5.4). Switching to an alternative antimicrobial (imipenem or meropenem) occurred on two occasions, of which one patient had a positive outcome and one was fatal. Trimethoprim-sulfamethoxazole was used in early therapy in 25/101(25%) iNTS; ceftriaxone was later added to this regime. Patients who were additionally diagnosed with *Talaromyces marneffe* or *Cryptococcus neoformans* in their BS or CSF were also treated with antifungal drugs. There was no significant difference in disease outcome with differing antimicrobial treatment regimens (Table 5.4). The median time from hospitalization to the use of an antimicrobial was 2.9 days (IQR 0-3 days); patients with a fatal outcome received an antimicrobial significantly earlier than those with non-fatal disease with a median of two days after hospitalization in the fatal group compared to 3.5 days in the non-fatal group ($p=0.01$; Kruskal-wallis test).

Table 5. 3 Covariates associated with fatal outcome in 102 patients with invasive non-typhoidal *Salmonella* disease

Characteristic	Univariable			Multivariable		
	OR	95%CI	p	aOR	95%CI	p
Male sex	1.13	0.42-2.99	0.813	0.82	0.23-2.95	0.757
Age category (yr) ^a						
<10	0.75	0.07-7.88	0.811	0.94	0.06-14.3	0.967
21-30	1.75	0.44-6.88	0.423	0.31	0.05-1.85	0.198
31-40	4.32	1.20-15.6	0.025	0.86	0.15-5.0	0.866
>40	1.00	-	-	1.00	-	-
HIV	8.31	1.8-38.1	0.006	7.89	1.10-56.3	0.039
Hepatitis	1.36	0.50-3.71	0.552	0.89	0.23-3.44	0.862
Serovar						
<i>S. Enteritidis</i>	3.08	0.76-12.4	0.114	2.91	0.65-12.9	0.161
<i>S. Typhimurium</i>	4.24	0.91-19.8	0.066	3.24	0.54-19.5	0.198
Other	1.00	-	-	1.00	-	-

OR: odds ratio; CI: confidence interval; aOR: adjusted odds ratio

a) No patients aged 10-20 years

Table 5. 4 The characteristics of antimicrobial treatment for invasive non-typhoidal *Salmonella* patients

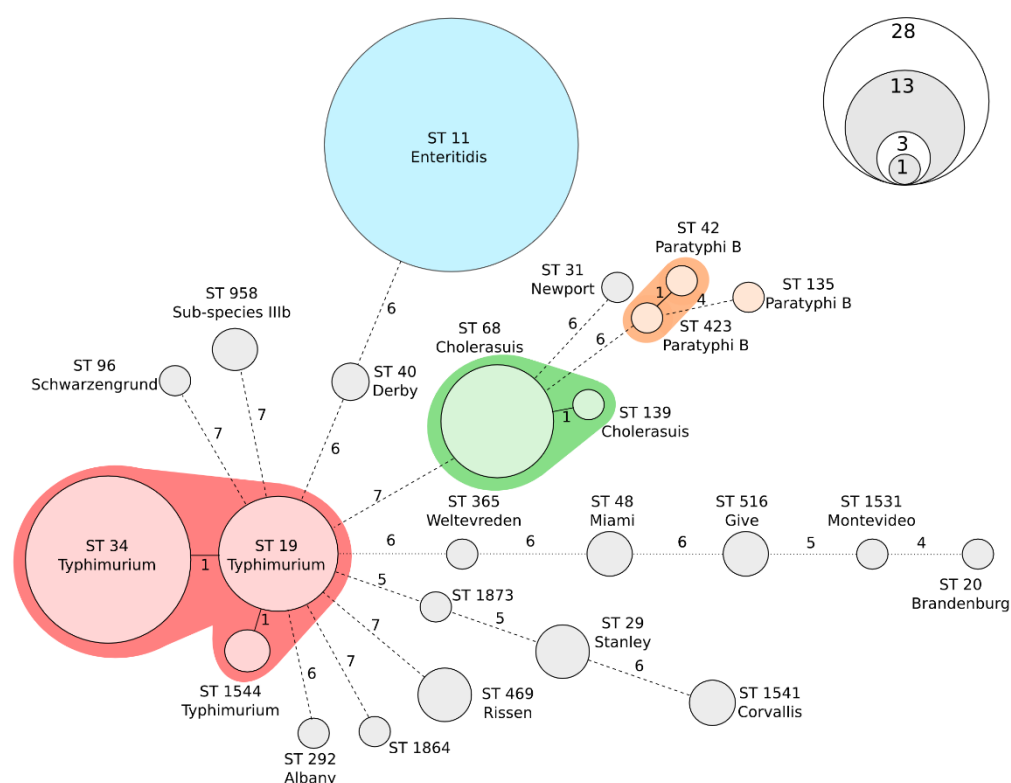
Antimicrobial prescribed	Count	Treatment, days		FCT, days ^a	
	n/Total (%)	n	Median (IQR)	n	Median (IQR)
Any	100/102 (98)	98	7 (3-10)	51	3 (2-8)
Ceftriaxone	89/100 (89)	89	7 (4-10)	47	3 (2-7)
Monotherapy	34/100 (34)				
Combination	55/100 (55)				
Fluoroquinolone	22/100 (22)	22	10 (7-14)	11	5 (2-10)
Monotherapy	7/100 (7)				
Combination	15/100(15)				
Combination therapy	53/100 (53)				
CRO-FLQ	16/100 (16)	15	10 (7-12)	9	4 (2-5)
CRO-SXT	25/100 (25)	25	6 (2-10)	6	2 (2-5)
Other	12/100 (12)				
Switched to a broader spectrum	4/101 (4)				

FCT: fever clearance time; a) non-fatal cases only; CRO-FLQ: ceftriaxone-fluoroquinolone; CRO-SXT: ceftriaxone- trimethoprim-sulfamethoxazole

5.3.1. Serovars associated with invasive non-typhoidal *Salmonella* infections

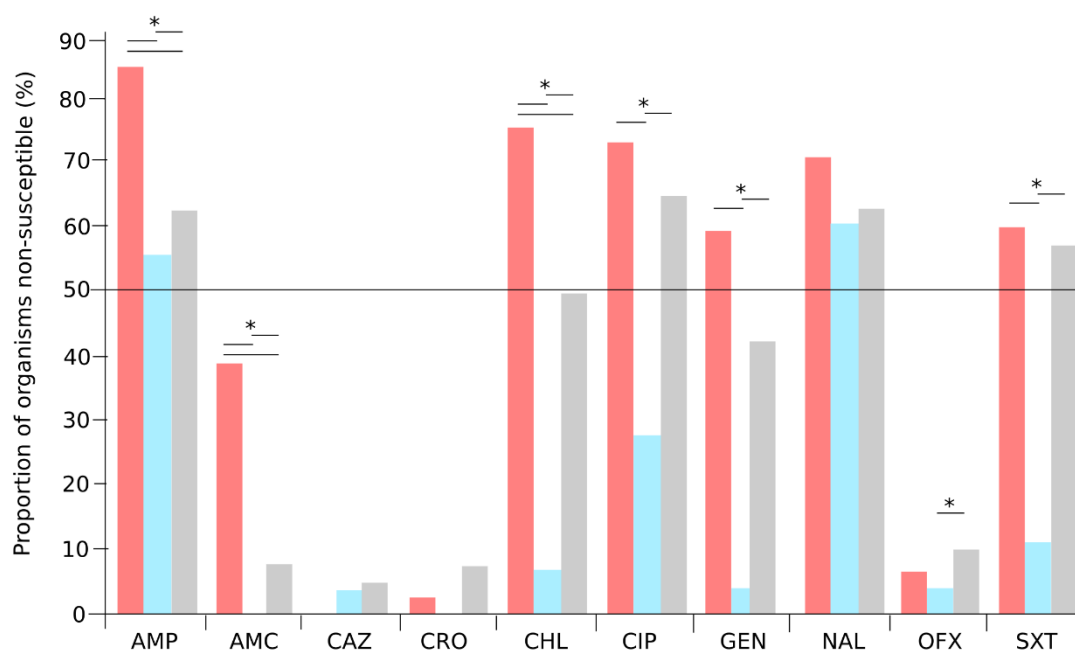
I performed MLST on the complete collection of 142 available iNTS isolates cultured at HTD between January 2008 and June 2013; the resulting minimum spanning tree of these data is shown in Figure 5.1. I was able to identify 17 different serovars by MLST that were associated with iNTS disease in this population. The most common serovars causing invasive disease were *S. Enteritidis* (ST11) and *S. Typhimurium* (STs 19, 34 and 1544), which were responsible for 63/147 (43%) and 44/147 (30%) of all cases, respectively. *S. Typhimurium* was identified more frequently in HIV-infected patients (Table 5.1) ($p=0.003$, Fisher's exact test). The remaining organisms ($n=40$) were a combination of less commonly isolated *Salmonella* serovars including *S. Choleraesuis* ($n=14$), *S. Stanley* ($n=3$) and *S. Weltevreden* ($n=1$) (Figure 5.1), which were generally identified in nonfatal cases.

Figure 5. 1 *Salmonella* serovars associated with invasive disease



Minimum spanning tree of 142 iNTS isolates created using seven allele MLST profiling. The sequence type (ST) of each allele profile is shown along with the inferred serovar. Clonal complexes (*S. Typhimurium*, *S. Enteritidis*, *S. Cholerasuis* and *S. Paratyphi B* [tartrate positive]) are highlighted. The size of each ST group corresponds with the number of isolates with the same ST profile (scale shown), and the branches are labeled by the number of variable alleles between STs.

Figure 5. 2 The antimicrobial susceptibility profiles of invasive *Salmonella*



Bar graph showing the proportion of organisms (red, *S. Typhimurium*; blue, *S. Enteritidis* and grey, others) exhibiting resistance against ampicillin (AMP), amoxicillin/clavulanate (AMC), ceftazidime (CAZ), ceftriaxone (CRO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), ofloxacin (OFX) and trimethoprim-sulfamethoxazole (SXT). Asterisks signify statistically significant differences in the proportion of organisms exhibiting resistance to the individual antimicrobial ($p < 0.05$, Fisher's exact test).

I next compared antimicrobial susceptibility profiles between *S. Enteritidis*, *S. Typhimurium* and the remaining serovars (Figure 5.2). The susceptibility profiles varied between the three different etiological groups and I found that *S. Typhimurium* were significantly more likely to exhibit resistance against ampicillin, amoxicillin, Chloramphenicol, and trimethoprim-sulfamethoxazole than *S. Enteritidis* and the remaining serovars ($p < 0.05$ for all pairwise comparisons, Fisher's exact test) (Figure 5.1). Further, >50% of *S. Typhimurium* isolates were resistant to 6/10 antimicrobials tested (including ciprofloxacin and gentamicin); the same was true for 2/10 tested antimicrobials with the *S. Enteritidis* isolates and 5/10 antimicrobials with the other iNTS isolates. The majority of iNTS were susceptible to both azithromycin and third generation cephalosporins, with the exception of a single Extended Spectrum Beta Lactamase (ESBL) producing *S. Choleraesuis*. After PCR amplification and sequencing I found this particular *S. Choleraesuis* isolate to harbor a *bla*_{CTX-M-55} ESBL gene (see chapter 4). Despite differences in AMR profiles I found no significant difference in mortality between those infected with *S. Typhimurium* and *S. Enteritidis* ($p = 0.431$, Fisher's exact test). Lastly, isolates from HIV-infected patients were significantly more likely to exhibit reduced susceptibility against trimethoprim-sulfamethoxazole (28/62 45%) compared to HIV-uninfected patients (6/28, 21%) ($p = 0.037$, Fisher's exact test).

5.4 Discussion

NTS pathogens are a leading cause of community acquired bloodstream infections in parts of sub-Saharan Africa (Crump and Heyderman, 2015; Feasey et al., 2012). In sub-Saharan Africa the disease is concentrated in children and HIV-infected adults and complicated by the recent emergence and dominance of ST313, a multidrug resistant (MDR) variant of *S. Typhimurium* (Okoro et al., 2012). It was not known whether similar epidemiological patterns and iNTS sequence types existed in Asia. I can report

that iNTS infections are not as common in this setting in comparison to parts of sub-Saharan Africa, but similarly the disease is associated with immunocompromised adults and primarily caused by the serovars *S. Enteritidis* and *S. Typhimurium*.

Recent evaluations in sub-Saharan Africa have highlighted that the emergence of iNTS has been largely driven in adults by the HIV epidemic, while malnutrition and malaria infection are heavily associated with iNTS in children (Crump and Heyderman, 2015; Feasey et al., 2012). The overall incidence of iNTS infections in Southeast Asia is limited compared to that of Africa (Gordon, 2011; Khan et al., 2010), though similar to the African context we confirm that HIV infection is the primary risk factor for iNTS disease in adults in Vietnam. The overall prevalence of HIV infection is low in Vietnam (0.5%) (UNAIDS, 2014), yet it is known that disease is common in adults using intravenous drugs (Go et al., 2011). Indeed, HIV positive individuals in this study were likely to be male between the ages of 28-37 years. Therefore, iNTS disease should be considered as a possible aetiology for febrile HIV-infected individuals.

As HIV was identified as main risk factor for iNTS diseases, I assume that HIV individuals should have convenient immune factors to NTS infection. In this case, bacterial killing assay is the appropriate method for better understanding of immune response to NTS in these immunocompromised patients (Gupta-Wright et al., 2017). Protocols for such assay in Salmonella including *S. Typhi*, *S. Paratyphi*, *S. Typhimurium* and *S. Enteritidis* had been developed for vaccine research (Boyd et al., 2014). My study had both HIV-infected iNTS and non-HIV infected iNTS patients. Using bacterial killing assay to compare the functioning of immune response in different groups of iNTS patients is a promising study in the future.

Through MLST testing I found that approximately 75% of iNTS organisms were either *S. Enteritidis* or *S. Typhimurium*, which is consistent with the organisms causing iNTS

disease in Africa (Feasey et al., 2015; Kariuki and Onsare, 2015), and the predominant organisms found in non-invasive NTS infections in this setting (Thompson et al., 2013). Although *S. Typhimurium* isolates were more likely to exhibit resistance against antimicrobials than other serovars, I did not identify the MDR *S. Typhimurium* clone ST313, which appears to have replaced resident NTS strains in sub-Saharan Africa (Okoro et al., 2012). The sequence types identified here setting, namely *S. Enteritidis* ST11 and *S. Typhimurium* ST19 and ST34, have been found in invasive infections in Africa previously (Ikumapayi et al., 2007; Kariuki and Onsare, 2015; Kingsley et al., 2009; Okoro et al., 2012) while *S. Typhimurium* ST1544 has recently been isolated from food samples from China (Yang et al., 2015). I additionally identified 14 *S. Choleraesuis* isolates, a serovar known to be associated with the consumption of pork products (Chiu et al., 2004), and previously shown to be a cause of bacteremia in Taiwan (Jean et al., 2006). I surmise that it is likely that organisms causing gastroenteritis in immunocompetent individuals may be comparable to those causing iNTS disease in immunocompromised patients in Vietnam. Continuing efforts to improve food safety and hygiene may have a positive effect on reducing both non-invasive and iNTS disease in this setting, though such interventions are costly and may be difficult to sustain in an industrializing setting like Vietnam (Unnevehr and Jensen, 1999).

Over one quarter of patients with iNTS disease either died in hospital or were discharged to die at home with family. This mortality rate is similar to the African context and confirms the severity of this infection in an immunocompromised population. The primary risk factor for death in our population was HIV infection, confirming trends identified in adults in sub-Saharan Africa (Crump and Heyderman, 2015; Feasey et al., 2012). Though I did not have CD₄ cell counts available, iNTS

disease is known to be a major risk factor for death in patients with advanced HIV disease (M. Gordon et al., 2002). As only 22% of HIV-infected patients were on active ART at the time of admission, improving access to ART would likely prevent the number of iNTS cases in Vietnam.

The majority of patients received ceftriaxone either in mono or combination therapy. Current susceptibility profiles confirm this is an appropriate choice, however high existing resistance against a variety of antimicrobials including ampicillin, chloramphenicol, and ciprofloxacin signal the propensity for *Salmonella* to acquire a variety of resistance mechanisms. High levels of antimicrobial resistance in *S. Typhimurium* is cause for concern, particularly as HIV-infected patients were most often diagnosed with this serovar and the presence of resistance could further complicate management. Attempting to identify whether such antimicrobial resistance is related to food consumption and the excessive use of antimicrobials in animal husbandry known to occur in Vietnam is warranted (Carrique-Mas et al., 2015). Furthermore, the significant elevation of trimethoprim-sulfamethoxazole resistance amongst HIV-infected patients suggests that pneumocystis prophylaxis with the drug leads to colonization by resistant organisms. These data indicate that reduced antimicrobial susceptibility may not purely arise in animals in zoonotic pathogens, further work regarding the use of specific antimicrobials in animals is justified.

Our study has limitations. First, children with HIV are generally referred to one of two large local pediatric hospitals so it is likely the burden of iNTS disease in children substantially underestimated. Though HIV is a risk factor for iNTS in children in Kenya (Muthumbi et al., 2015), malnutrition and malaria infection are also important risks in children in the sub-Saharan African context (Berkley et al., 2005; Graham et al., 2000); future work in an Asian context should examine the epidemiology of pediatric iNTS

more thoroughly. Secondly, this retrospective analysis for risk of death may be biased by misclassification as we coded patients who were taken home by family members as fatal, though I did not have a confirmed death report from these individuals.

Notwithstanding these limitations of a retrospective study this work provides the largest description to date of iNTS patients to date in Southeast Asia and highlights important similarities and differences between the African and Asian settings. I suggest that continued surveillance, including sequence typing/whole genome sequencing, should be performed to monitor for emergence or introduction of MDR strains or strains with any apparent enhanced virulence phenotype, such as ST313 (Nguyen et al., 2013).

I conclude that iNTS disease is a severe infection in Vietnam, with a mortality rate (26%) similar to that of sub-Saharan Africa. I also highlight HIV infection as the major risk for both infection and death in this setting. Though the sequence types of iNTS organisms identified in this study are common globally, I suggest continued surveillance to monitor for the presence of MDR sequence types, such as ST313, which has not, as of yet, been identified in Asia.

Chapter 6

A descriptive study of antimicrobial resistance in *Salmonella* spp. and a comparison between differing susceptibility testing methods

6.1 Aims of chapter

This study has two main aims. The first aim was to examine the trend of antimicrobial susceptibility of all *Salmonella* spp. isolated from blood cultures within an eight-year period (2008-2015) at HTD. The data presented in chapter 3 demonstrated that there was notable change in type of *Salmonella* associated with bacteraemia in HTD between 2010 and 2014. I observed an increase number in NTS BSI, as opposed *S. Typhi* being the predominant serovar (Nga et al., 2012). Moreover, there was an increase in AMR in Gram-negative bacilli associated with BSI (as described in chapter 3); this was predominantly associated with beta-lactams and fluoroquinolones. These two antimicrobial groups are the first line therapeutics for typhoid and NTS infections in HTD. Therefore, in this study, I will describe the susceptibility trends for antimicrobials for *Salmonella* BSI by the E-test method and the disk diffusion method. Based on the MIC of each antimicrobial obtained by E-test, the MIC₅₀ and MIC₉₀ will be determined for defined antimicrobials in *Salmonella* population. The distribution of MICs is valuable for epidemiology investigation and empirical treatment guidelines.

I additionally aimed to identify the most appropriate antimicrobial testing method for *Salmonella*. The E-test method is considered as the best antimicrobial susceptibility method for clinical microbiology but it is expensive. The disk diffusion method is historically the most common approach but it is unable to produce MIC values. Additionally, new automatic antimicrobial susceptibility testing machines, such as VITEK, have become increasingly popular Vietnam. In this study, I compare the accuracy and agreement level of three antimicrobial susceptibility testing methods (E-

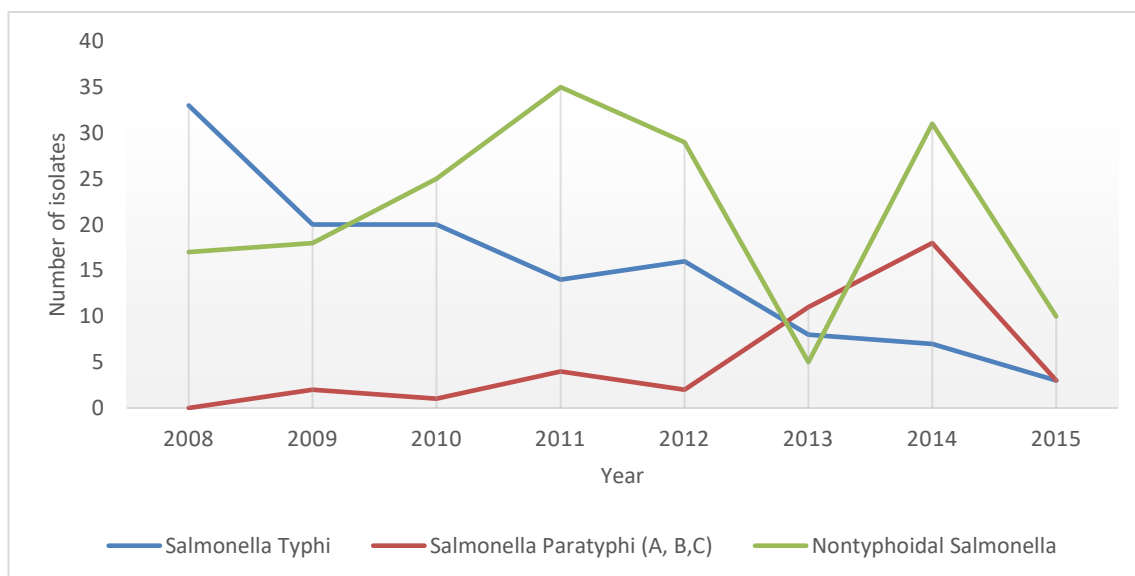
test, VITEK, and disk diffusion) for common antimicrobials used to treat infections caused by *Salmonella* sp. Data were analysed according to ISO 20776:2007 and CLSI M100: 2016 guidelines.

6.2 Results

6.2.1 *Salmonella* organisms and prevalence

Among 317 *Salmonella* isolated over the study period, 117 (36.9%) were *S. Typhi*, 23 (7.3%) were *S. Paratyphi* A, 8 (2.5%) were *S. Paratyphi* B, and 168 (53%) were NTS (Figure 6.1). It was notable that the number of *S. Typhi* being isolated steadily decreased over this period. NTS were more commonly isolated than *S. Typhi* from 2009, with the exception of a fall in NTS cases in 2013. 2013 was the time that HTD stopped receiving all HIV patients, and only received moderate or severe cases. The rise of NTS cases in 2014 was associated with hepatitis in non-HIV infected patients.

Figure 6. 1 Trend of different types of *Salmonella* associated with bacteraemia at HTD over an 8-year period (2008-2015)



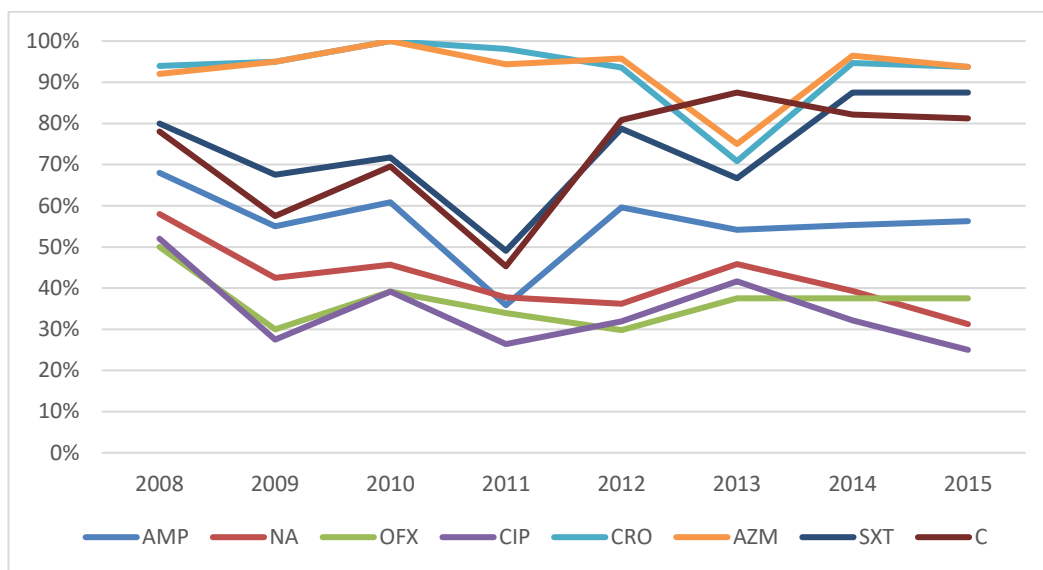
Line chart describing the actual number of *Salmonella* isolated per year divided into three main groups: *Salmonella* Typhi (blue line), *Salmonella* Paratyphi (including serotype A, B and C) (orange line) and nontyphoidal *Salmonella* (grey line).

6.2.2 Trends of antimicrobial susceptibility by E-test method

The trends of antimicrobial susceptibility of 332 *Salmonella* isolates are shown in Figure 6.2. The (fluoro)quinolone (nalidixic, ofloxacin, and ciprofloxacin) were the antimicrobials to which the organisms were least susceptible (annual susceptibility rate <50%), followed by the beta-lactam, ampicillin (55.2%). Susceptibility against chloramphenicol increased over the study period. Third generation cephalosporins (ceftriaxone) and azithromycin exhibited the highest susceptible rate across all *Salmonella* (>90%) (Figure 6.2). The older generation antimicrobials (ampicillin, trimethoprim-sulfamethoxazole and chloramphenicol) exhibited a similar trend: decreased susceptibility from 2009 to 2011, then regaining susceptibility after this period.

The distribution of MIC₅₀ and MIC₉₀ in common antimicrobials used to treat infections caused by *Salmonella* spp. is shown in Table 6.1. I found that nalidixic acid; an early generation of fluoroquinolones, was the least susceptible antimicrobial against *Salmonella*. The proportion of resistant organisms against nalidixic was 55.2% (175/317). MIC testing showed that 50% (158/317) of *Salmonella* had an azithromycin MIC value of 256µg/ml, this was most apparent in the *S. Typhi* and the NTS. Moreover, resistance was observed for approximately 10% of the fluoroquinolones (35/317; 11% for ofloxacin and 30/317; 9.5% for ciprofloxacin). Ciprofloxacin had lower MIC₅₀ and MIC₉₀ values than ofloxacin in both the *S. Typhi* and the NTS strains. The MIC₉₀ value for ciprofloxacin was ≤0.75 µg/ml in the *Salmonella* population, which implicated the majority of organisms in the susceptible and intermediate range.

Figure 6. 2 Susceptibility trends of several antimicrobials for 332 *Salmonella* isolated from blood between 2008 and 2015



AMP: ampicillin; NA: nalidixic acid; OFX : ofloxacin; CIP: ciprofloxacin; CRO: ceftriaxone; AZM: azithromycin; SXT: trimethoprim-sulfamethoxazole: Susceptible by using E-test method ; C: chloramphenicol (susceptibility was performed by the disk diffusion method)

Table 6. 1 MIC and susceptibility pattern of 317 *Salmonella* isolates by E-test method

	MIC ₅₀ (MIC ₉₀) (µg/ml)						
	Nalidixic acid	Ofloxacin	Ciprofloxacin	Azithromycin	SXT	Ampicillin	Ceftriaxone
<i>Salmonella</i> Typhi (n=117)	256 (256)	0.75 (1.0)	0.38 (0.75)	8.0 (12.0)	0.023 (32.0)	0.75 (256)	0.25 (0.25)
<i>Salmonella</i> Paratyphi (n=32)	4.0 (256)	0.19 (2.0)	0.064 (0.5)	8.0 (16.0)	0.094 (0.12)	1.5 (256)	0.25 (0.25)
Nontyphoidal <i>Salmonella</i> (n=168)	256 (256)	0.5 (2.0)	0.125 (0.5)	6.0 (8.0)	0.19 (32.0)	256 (256)	0.12 (0.19)
All <i>Salmonella</i> (n=317)	256 (256)	0.5 (1.5)	0.125 (0.5)	6.0 (12.0)	0.094 (32)	256 (256)	0.19 (0.25)
MIC range	0.5-256	0.016-32	0.004-32	1.5-256	0.008-32	0.25-256	0.016-32.0
Resistance proportion %	55.2	11	9.5	1.3	21.8	42	1.6

*MIC: minimum inhibition concentration. SXT: trimethoprim -sulfamethoxazole

I found that resistance against ampicillin was high at 42% (133/317), which made ampicillin the second most common antimicrobial that *Salmonella* were resistant to over the 8-year period. All three of the subgroups of *Salmonella* had high ampicillin MIC₉₀ value at 256 µg/ml. However, *Salmonella* had high susceptibility to ceftriaxone. Low MIC₅₀ and MIC₉₀ value at <0.25 µg/ml for ceftriaxone were observed in majority of *Salmonella* strains. Only five isolates were found to be resistant to ceftriaxone, all of which were isolated after 2011. One ceftriaxone-resistant organism was *S. Typhi*, the remainder belonged to NTS group.

Azithromycin, the oral azalide drug maintained its susceptibility for *Salmonella* over the 8 years. Resistance was identified in only four *Salmonella*, whose MIC value were >32µg/ml. All MIC₉₀ in all *Salmonella* subgroups was less than or equal to the susceptible cut-off (<16 µg/ml). The NTS organisms had lower MIC₅₀ than *S. Typhi* (6.0µg/ml vs. 8.0µg/ml).

Approximately, 22% (69/317) of *Salmonella* isolates were resistant to trimethoprim-sulfamethoxazole (SXT) as determined by E-test method. The SXT MIC₅₀ value in the NTS group was 0.19 µg/ml, the highest MIC₅₀ of this drug in all species.

6.2.3 Multidrug-resistant *Salmonella*

53/332 *Salmonella* was categorized as being multi-drug resistant (MDR; resistant to ampicillin, chloramphenicol, and SXT). There was an increasing trend of MDR isolation from 8.5% (4/47) to 38.5% (20/52) during 2008 -2011 (Table 6.2). After 2011, the MDR rate declined unevenly and varied between 5% and 13%. More than 70% of MDR strains were NTS. MDR *S. Typhi* was recorded for 12 isolates only all were cultured before 2012.

Table 6. 2 The prevalence of MDR *Salmonella* from 2008-2015

Year	2008	2009	2010	2011	2012	2013	2014	2015	Total
No of MDR strains	4	7	10	20	5	1	4	2	53
% of MDR	8.5	18.4	21.7	38.5	11.1	5.5	7.3	12.5	16.7
MDR ST (%)	3	0	2	5	2	0	0	0	12(22.6)
MDR SP (%)	0	0	1	1	0	0	0	0	2(3.8)
MDR NTS (%)	1	7	7	14	3	1	4	2	39 (73.6)

*MDR: multi-drug resistant (co-resistant against ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole);

ST: *Salmonella* Typhi; SP: *Salmonella* Paratyphi; NTS: nontyphoidal *Salmonella*

6.2.4 A comparison of three antimicrobial testing methods

I compared three commonly used clinical antimicrobial testing methods: E-test, an automated VITEK system, and disc diffusion to test seven antimicrobials against all 317 *Salmonella*. The seven-selected antimicrobials were ampicillin, ceftriaxone, nalidixic acid, ofloxacin, ciprofloxacin, azithromycin, and trimethoprim-sulfamethoxazole. They are all appropriate antimicrobials for *Salmonella* infections. Chloramphenicol was not selected as it is not incorporated into a VITEK screening card and the chloramphenicol E-test is not available in Vietnam. Because nalidixic acid, ofloxacin and azithromycin were not represented on the VITEK AST GN67 card, a comparison between VITEK method and E-test was not possible for these antimicrobials. Table 6.3 shows a comparison result for all *Salmonella* isolates collected over 8 years. Table 6.4, Table 6.5, and Table 6.6 describe a comparison for the various *Salmonella* subgroups (*S. Typhi*, *S. Paratyphi* (A, B, and C), and NTS, respectively).

For nalidixic acid, categorical agreements (susceptible, intermediate, and resistant) between E-test and disk diffusion method was found at high rate (>90%; 286/317) for all *Salmonella*. The *S. Paratyphi* isolates had highest agreement rate at 97% (31/32) for nalidixic acid by the two methods. The *S. Typhi* and NTS subgroups had approximately 90% category agreement by MIC E-test and disk diffusion. Minor error was very low (1/317, 0.3%), which occurred the in *S. Paratyphi* organisms only. The major error for nalidixic acid was as high as 13.4% (19/142) which was higher than the acceptable rate (<3%). 18/19 organisms that had major error in nalidixic acid were mainly in the NTS group. Very major error rate of disk diffusion method was 6.3% (11/175) of organisms in comparison with MIC E-test method), which was also higher than acceptable value (<1.5%). 10/11 strains that were susceptible to nalidixic acid by disk diffusion and

resistant by MIC E-test (very major error) were all *S. Typhi*. *S. Paratyphi* and NTS had very low very major errors for nalidixic acid in comparison with disk diffusion and MIC E-test.

Ofloxacin had low overall categorical agreement (126/317; 39.7%) between E-test and disk diffusion method for the *Salmonella* isolates in this study. Minor error ranged from 48% -60% in different *Salmonella* groups. However, there was no major error, which means all resistant strains against ofloxacin by disk diffusion, were also resistant by MIC E-test. In contrast, very major error was found to be very high (88%). 5/5 (100%) *S. Typhi*, 3/5 (60%) *S. Paratyphi* and 23/25 (92%) NTS strains were susceptible by MIC E-test method but found to be non-susceptible by disc diffusion method.

I found that the automated susceptibility card AST GN69 failed to differentiate between ciprofloxacin susceptible and intermediate organisms. The lowest dilution of ciprofloxacin was 0.5µg/ml on the VITEK card, which covered the susceptible (<0.06 µg/ml) and intermediate range (0.12-0.5 µg/ml). Therefore, the minor error and the very major error rate were not obtainable. However, I could assess the major error rate by assessing resistance to ciprofloxacin by MIC VITEK but susceptible by E-test. A major error was found on only NTS organism. In a comparison between MIC E-test and disk diffusion for ciprofloxacin, categorical agreement was low at 54% (171/317). The disk diffusion method provider a high minor error (41.2%, 141/317) and very major error (20%, 6/30) rate. Minor error of ciprofloxacin was detected in all *Salmonella* groups, while the major error arose in 6 NTS organisms only.

Ampicillin, an older generation antimicrobial used to treat *Salmonella*, exhibited good agreement between the different antimicrobial testing methods. MIC VITEK showed good categorical interpretation agreement (> 99%) with the MIC E-test method for

every *Salmonella* group. There was only one NTS organism that was identified as resistant to ampicillin by VITEK method but susceptible by E-test method. Moreover, the ampicillin testing result by disk diffusion also exhibited a good correlation with the E-test method. Categorical agreement for ampicillin was 97.8% (310/317) for disk diffusion and E-test methods. Very major error was found only in one NTS isolated while a major error was recorded for three *S. Typhi* and one NTS isolate. In general, ampicillin had good interpretation agreement between disk diffusion and MIC VITEK method. Ceftriaxone demonstrated a comparable result to ampicillin. The MIC VITEK methods produced identical results in comparison with E-test for ceftriaxone. 315/317 (99.4%) of the *Salmonella* isolated had the same interpretation result by disk diffusion and MIC E-test for ceftriaxone.

Azithromycin was compared by the MIC E-test method and the disk diffusion method only. 292/317 (92%) organisms had a good correlation by these two testing methods. Although azithromycin had very few minor and major errors (only one for each error), there was more major error (23/317; 7.4%) when compared to the disk diffusion method. 13/23 cases with a major error were NTS, 6 were *S. Typhi* and four were *S. Paratyphi*.

SXT was tested by all three methods. VITEK proved to be a good testing method for *Salmonella* with 97.5% (309/317) in agreement with the E-test method. There were six minor errors (mostly NTS), one major, and one very major error. With the VITEK method, disk diffusion method had 95.6% (303/317) agreement with VITEK interpretation. Minor and major errors were under the specified cut-offs. However, 3/73 (4.3%) organisms produced a susceptible result by disk diffusion but were susceptible by E-test method (interpreted as very major error); these were all *S. Typhi*.

Table 6. 3 Comparison between antimicrobials testing methods for 317 *Salmonella* isolated during 2008-2015

	No.(%) OF ISOLATES			No.(%) OF ISOLATES			
	Susceptible	Intermediate	Resistant	Categorical agreement with E-test method	Minor error	Major error	Very major error
Nalidixic acid*							
E-TEST	142 (44.8)	0	175 (55.2)				
DISK DIFFUSION	133 (42)	1 (0.3)	183 (57.7)	286 (90.2)	1 (0.3)	19 (13.4)	11 (6.3)
Ofloxacin*							
E-TEST	123 (38.8)	159 (50.2)	35 (11)				
DISK DIFFUSION	311 (98.1)	3 (0.95)	3 (0.95)	126 (39.7%)	160 (50.5)	0	31 (88.6)
Ciprofloxacin							
E-TEST	116 (36.6)	171 (53.9)	30 (9.5)				
VITEK2	NA**	NA**	51 (55.4)	NA**	NA**	0	NA**
DISK DIFFUSION	194 (61.2)	91 (28.7)	32 (10.1)	171(53.9)	140 (41.2)	0	6 (20)
Ampicillin							
E-TEST	184 (58)	0	133 (42)				
VITEK2	183 (57.7)	0	134 (42.3)	316 (99.7)	0	1 (0.5)	0
DISK DIFFUSION	179(56.5)	2 (0.6%)	136 (42.9)	310 (97.8)	2(0.6)	4 (2.2)	1 (0.8)
Ceftriaxone							
E-TEST	312 (98.4)	0	5 (1.6)				
VITEK2	312 (98.4)	0	5 (1.6)	317 (100)	0	0	0
DISK DIFFUSION	311 (98.1)	1 (0.3)	5 (1.6)	315 (99.4)	1 (0.3)	1 (0.3)	0
Azithromycin*							
E-TEST	312 (98.4)	1 (0.3)	4 (1.3)				
DISK DIFFUSION	290 (91.5)	0	27 (8.5)	292 (92.1)	1 (0.3)	23 (7.4)	1 (25)
Trimethoprine-sulfamethoxazole							
E-TEST	242 (76.3)	6 (1.9)	69 (21.8)				
VITEK2	244 (77%)	6 (1.9)	67 (21.1)	309 (98)	6 (1.9)	1 (0.4)	1 (1.4)
DISK DIFFUSION	242 (76.3%)	2 (0.6)	73 (23)	303 (96)	8 (2.5)	3 (1.2)	3 (4.3)

*VITEK results were unavailable for nalidixic acid, ofloxacin and azithromycin.

**Interpretation result in ciprofloxacin susceptible (<0.06 µg/ml) and intermediate (0.12-0.5 µg/ml) were not available (minimum VITEK MIC for ciprofloxacin was <0.5µg/ml). Bold number implicated the value that was higher than accepted range.

Table 6. 4 Comparison between antimicrobials testing methods for 117 *Salmonella* Typhi isolated during 2008-2015

	No.(%) OF ISOLATES			No.(%) OF ISOLATES			
	Susceptible	Intermediate	Resistant	Categorical agreement with E-test method	Minor error	Major error	Very major error
Nalidixic acid*							
E-TEST	41 (35)	0	76 (65)				
DISK DIFFUSION	50 (42.7)	0	67 (57.3)	106 (90.5)	0	1 (2.4)	10 (13.1)
Ofloxacin*							
E-TEST	42 (35.9)	70 (59.8)	5 (4.3)				
DISK DIFFUSION	117 (100)	0	0	42 (35.9)	70 (59.8)	0	5 (100)
Ciprofloxacin							
E-TEST	40 (34.19)	59 (50.4)	18 (15.4)				
VITEK2	NA**	NA**	33 (56.9)	NA**	NA**	0	NA**
DISK DIFFUSION	20 (17.1)	71 (60.7)	26 (22.2)	75 (64)	42 (35.9)	0	0
Ampicillin							
E-TEST	99 (84.6)	0	18 (15.4)				
VITEK2	99 (84.6)	0	18 (15.4)	117 (100)	0	0	0
DISK DIFFUSION	94 (80.3)	2 (1.7)	21 (18)	112 (95.7)	2 (1.7)	3 (3)	0
Ceftriaxone							
E-TEST	117 (100)	0	0				
VITEK2	117 (100)	0	0	117 (100)	0	0	0
DISK DIFFUSION	117 (100)	0	0	117 (100)	0	0	0
Azithromycin*							
E-TEST	116 (99.1)	1 (0.9)	0				
DISK DIFFUSION	110 (94)	0	7 (6)	110 (94)	1 (0.9)	6 (5.1)	0
Trimethoprim-sulfamethoxazole							
E-TEST	99 (84.6%)	0	18 (15.4)				
VITEK2	99 (84.6%)	0	18 (15.4)	115 (98.3)	0	1 (1%)	1 (5.6)
DISK DIFFUSION	102 (87.2%)	0	15 (15.4)	114 (97.4)	0	0	3 (16.7)

*VITEK results were unavailable for nalidixic acid, ofloxacin and azithromycin.

**Interpretation result in ciprofloxacin susceptible (<0.06 µg/ml) and intermediate (0.12-0.5 µg/ml) were not available (minimum VITEK MIC for ciprofloxacin was <0.5µg/ml). Bold number implicated the value that was higher than accepted range.

Table 6. 5 Comparison between antimicrobials testing methods for 32 *Salmonella* Paratyphi isolated during 2008-2015

	No.(%) OF ISOLATES			No.(%) OF ISOLATES			
	Susceptible	Intermediate	Resistant	Categorical agreement with E-test method	Minor error	Major error	Very major error
Nalidixic acid*							
E-TEST	23 (71.9)	0	9 (28.1)				
DISK DIFFUSION	22 (68.8)	1 (3.13%)	9 (28.1)	31 (96.9)	1 (3.1)	0	0
Ofloxacin*							
E-TEST	18 (56.3)	9 (28.1)	5 (15.6)				
DISK DIFFUSION	29 (90.6)	1 (3.13)	2 (6.3)	19 (59.4)	10 (31.3)	0	3 (60)
Ciprofloxacin							
E-TEST	21 (65.6)	9 (28.1)	2 (6.3)				
VITEK2	NA**	NA**	5 (83)	NA**	NA **	0	NA**
DISK DIFFUSION	28 (87.5)	2 (6.3)	2 (6.3)	25 (78.1)	7 (21.9)	0	0
Ampicillin							
E-TEST	26 (81.3)	0	6 (18.8)				
VITEK2	26 (81.3)	0	6 (18.8)	31(96.7)	0	0	0
DISK DIFFUSION	26 (81.3)	0	6 (18.8)	32 (100)	0	0	0
Ceftriaxone							
E-TEST	30 (93.8)	0	2 (6.3)				
VITEK2	30 (93.8)	0	2 (6.3)	32 (100)	0	0	0
DISK DIFFUSION	30 (93.8)	0	2 (6.3)	32 (100)	0	0	0
Azithromycin*							
E-TEST	31 (96.9)	0	1 (3.1)				
DISK DIFFUSION	27 (84.4)	0	5 (15.6)	28 (87.5)	0	4 (12.9)	0
Trimethoprine-sulfamethoxazole							
E-TEST	28 (87.5)	1 (3.1)	3 (9.4)				
VITEK2	29 (90.6)	0	3 (9.4)	31 (96.9)	1 (3.1%)	0	0
DISK DIFFUSION	29 (90.6)	0	3 (9.4)	31 (96.9)	1 (3.1%)	0	0

*VITEK results were unavailable for nalidixic acid, ofloxacin and azithromycin.

**Interpretation result in ciprofloxacin susceptible (<0.06 µg/ml) and intermediate (0.12-0.5 µg/ml) were not available (minimum VITEK MIC for ciprofloxacin was <0.5µg/ml). Bold number implicated the value that was higher than accepted range.

Table 6. 6 Comparison between antimicrobials testing methods for 168 non-typhoidal *Salmonella* isolated during 2008-2015

	No.(%) OF ISOLATES			No.(%) OF ISOLATES			
	Susceptible	Intermediate	Resistant	interpretive agreement with the E-test method	Minor error	Major error	Very major error
Nalidixic acid*							
E-TEST	78 (46.4)	0	90 (53.6)				
DISK DIFFUSION	61 (36.3)	0	107 (63.7)	150 (89.3)	0	18 (23.1)	1 (1.1)
Ofloxacin*							
E-TEST	63 (37.5)	80 (47.6)	25 (14.9)				
DISK DIFFUSION	165 (98.2)	2 (1.2)	1 (0.6)	65 (38.7)	80 (47.6)	0	23 (92)
Ciprofloxacin							
E-TEST	76 (45.2)	86 (51.2)	6 (3.6)				
VITEK2	NA***	NA***	13 (46.4)	NA**	NA**	1 (1.3)	NA**
DISK DIFFUSION	146 (86.9)	18 (10.7)	4 (2.4)	71 (42.3)	91 (54.2)	0	6 (100)
Ampicillin							
E-TEST	59 (35.1)	0	109 (64.9)				
VITEK2	58 (34.5)	0	110 (65.5)	167 (99.4)	0	1 (1.7)	0
DISK DIFFUSION	58 (34.5)	0	110 (65.5)	167 (99.4)	0	1 (1.7)	0
Ceftriaxone							
E-TEST	166 (98.8)	0	2 (1.2)				
VITEK2	166 (98.8)	0	2 (1.2)	168 (100)	0	0	0
DISK DIFFUSION	164 (97.6)	1 (0.6)	3 (1.8)	166 (98.8)	1 (0.6)	1 (0.6)	0
Azithromycin*							
E-TEST	165 (98.3)	0	3 (1.8)				
DISK DIFFUSION	153 (91)	0	15 (8.9)	154 (91.7)	0	13 (7.9)	1 (33.3)
Trimethoprine-sulfamethoxazole							
E-TEST	115 (68.5)	5 (3)	48 (28.6)				
VITEK2	116 (69.1)	6 (3.6)	46 (27.4)	163 (97)	5 (3)	0	0
DISK DIFFUSION	111 (66.1)	2 (1.2)	55 (32.7)	160 (95.2)	7 (4.2)	1 (0.9)	0

*VITEK results were unavailable for nalidixic acid, ofloxacin and azithromycin.

**Interpretation result in ciprofloxacin susceptible (<0.06 µg/ml) and intermediate (0.12-0.5 µg/ml) were not available (minimum VITEK MIC for ciprofloxacin was <0.5µg/ml). Bold number implicated the value that was higher than accepted range.

6.3 Discussion

This study was the first study to assess the performance of common AST methods for bacteria in Vietnam. This work was focussed on *Salmonella*, a commonly isolated member of the Enterobacteriaceae, which can cause typhoid fever or BSI.

Decreased susceptibility against fluoroquinolone has been reported in developed and developing countries. Decreased susceptibility to ciprofloxacin rose from 35% in 2001 up to 70% in 2007 in the United Kingdom (Crump et al., 2015). A very high prevalence (15%-46%) of reduced susceptibility to ciprofloxacin was identified in NTS isolated in Southeast Asia; Thailand reported 31% resistance against ciprofloxacin in NTS isolates (Wannaprasat et al., 2011). In this study, I found that fluoroquinolones (including nalidixic acid, ofloxacin, and ciprofloxacin), a key group of drugs for *Salmonella* infection had low susceptibility rate in this study, using CLSI interpretations. The resistance rate against nalidixic acid was 55.2% (35/317); 11% (35/317) for ofloxacin, and 9.5% (30/317) for ciprofloxacin. A previous study investigating AST profiles of *S. Typhi* between 2008-2009 in Vietnam showed that nalidixic acid resistant was 19.6%, but there was no resistance against ciprofloxacin (Chiou et al., 2014). My data between 2008 and 2014 demonstrates the rise of fluoroquinolone resistance in *Salmonella* in Vietnam, which has followed the same trend as in other countries. This observation suggests a requirement to evaluate the clinical effect of fluoroquinolones for treating *Salmonella* infections.

Reduced susceptibility against fluoroquinolones is associated with specific chromosomal mutations in NTS. Resistance to ciprofloxacin was associated with a mutation in the *gyrA* gene in isolates from the Congo; mutations in *gyr*, *par* and *snr* genes in Bangladesh, and *gyrA* and *par* genes in the US (Chiou et al., 2014; Lunguya et

al., 2013). In Vietnam, nalidixic resistance due to mutations in *gyrA* had been reported in *S. Typhi* from 1993(Wain et al., 1997). Recently, plasmid mediated-fluoroquinolone-resistant genes (*qnrS*, *qnrB* and *qnrS*), had been described in Enterobacteriaceae isolated from faecal samples from Vietnamese children in 2012(Vien et al., 2012). It would be interesting to identify the mechanism of fluoroquinolone resistance in *Salmonella* isolates associated with BSI in this location.

A multinational study from Asia (Korea, Thailand and Taiwan) revealed that NTS had decreased susceptibility against ciprofloxacin with MIC₅₀ (0.12 µg/ml) and MIC₉₀ (1-4 µg/ml)(Lee et al., 2009). The NTS organisms in my study had the same MIC₅₀ (0.12 µg/ml) and lower MIC₉₀ (0.25 µg/ml) to ciprofloxacin than this previous study. Another recent study originating from the Middle East and central Asia found decreased susceptibility of ciprofloxacin in *S. Typhi* (Rahman et al., 2014), which was comparable to the *S. Typhi* isolates in this study (MIC₅₀/MIC₉₀: 0.38/0.75 µg/ml). However, the MIC₅₀ (256 µg/ml) and MIC₉₀ (256 µg/ml) for ampicillin in my study were higher than other countries in Asia (MIC₅₀ 0.5-1.0 µg/ml, MIC₉₀ >126 µg/ml). This result suggests the threat of potential MDR NTS epidemic in Vietnam. However, the ceftriaxone MIC₅₀ (0.12 µg/ml) and MIC₉₀ (0.19 µg/ml) in my study was equivalent to that from other Asian nations(Lee et al., 2009). In my study, 53/317 *Salmonella* isolates were MDR, in which 74% (39/53) were NTS. A study from Malawi found that 59% of NTS in BSI had an MDR phenotype (Feasey et al., 2015) . One notable finding was that MDR *S. Typhi* was isolated before 2013 only. This marked a significant shift of the *S. Typhi* antimicrobial profile, because 70% of *S. Typhi* were MDR between 1993 and 1994 (Hoa et al., 1998).

Here I did not use the broth micro dilution method (BMD), which is the standard method for MIC testing; I alternatively used E-test, a clinical AST method as a

standard. BMD is not a practical method for a clinical microbiology laboratory and a good agreement between E-test and BMD has been shown in many studies. In early 1990s, Baker *et al.* reported good categorical agreement of >92% between E-test and BMD for fluoroquinolones and beta-lactams for Gram-negative rods (Baker et al., 1991). Even for colistin or other polymyxins, E-test exhibited nearly 90% agreement with the reference BMD method (Heijden et al., 2007) .

It was not possible to procure a VITEK AST card that contained all the required antimicrobial for *Salmonella* susceptibility testing, especially nalidixic acid, chloramphenicol and azithromycin. Therefore, testing these three antimicrobials was not possible by automated VITEK methods. Further, the VITEK AST card did not have the interpretation cut-off for the range 0.06-0.5 µg/ml for ciprofloxacin, therefore I was unable to determine whether *Salmonella* isolates with MIC <0.5 µg/ml for ciprofloxacin were resistant or intermediate. However, I found very good correlation % between VITEK result and E-test for all tested beta-lactams. Trimethoprim-sulfamethoxazole also had a good correlation between E-test and VITEK. In my opinion, the VITEK AST GN 69 card did not appropriately test *Salmonella* due to limited antimicrobials and provide insufficient interpretation for ciprofloxacin, although it did have a good performance for beta-lactams and trimethoprim-sulfamethoxazole.

Poor categorical agreement between disk-diffusion and E-test result was found for the fluoroquinolones. Low categorical agreement was caused by high minor errors of 50.5% (160/317) for ofloxacin and 41.2% (140/317) for ciprofloxacin. Since minor error could possibly result from the technical practice, repeated testing should be performed for ofloxacin and ciprofloxacin by disk diffusion method. Moreover, I also found a high rate of very major error for these two antimicrobials in the NTS isolates, which means

disk diffusion test failed to identify resistance in these group. I recommend that ofloxacin and ciprofloxacin should be tested by E-test for better precision for NTS. Disk diffusion provided good ability, in comparison with E-test for beta-lactams, azithromycin and SXT. This means that disk diffusion, a simple and manual technique, is a reliable testing method for these antimicrobials for *Salmonella*. In Vietnam, except for some central microbiology laboratories, disk diffusion is still the method of choice for antimicrobial susceptibility testing. With my results, I propose that clinical microbiology laboratories should continue to use the disk-diffusion method to test beta-lactams, azithromycin, and SXT.

I additionally found that chloramphenicol and trimethoprim -sulfamethoxazole regained their effectively in the latter stages of the study. The appearance of five *Salmonella* organisms exhibiting resistance to ceftriaxone highlights the emergence of NTS organism with ESBL activity. Therefore, chloramphenicol and SXT could be considered to treat *Salmonella* infections for the purpose of limiting the usage of third generation cephalosporins.

In conclusion, the MIC VITEK and disk diffusion exhibited good performance for testing beta-lactams, ampicillin and ceftriaxone. MIC E-test and disk diffusion methods gave a good comparison result for SXT, but gave some very major errors for *S. Typhi* only. Azithromycin had good categorical agreement between disk diffusion test and MIC E-test. Problematic agreement or several errors were found with the quinolones (nalidixic acid, ofloxacin, ciprofloxacin). The VITEK automatic susceptibility testing system did not appropriately test for *Salmonella* as it was unable to generate breakpoints for ciprofloxacin, it was missing nalidixic, ofloxacin and azithromycin, although it had good performance with beta-lactams. The disk-diffusion method is likely the most

reliable method for testing beta-lactams, azithromycin and trimethoprim-sulfamethoxazole.

Chapter 7

Conclusion and future research direction

After performing my PhD research on BSI in HTD, I have outlined some conclusions and final thoughts:

1. BSIs in HTD were associated with both bacterial and fungal pathogens.

BSIs associated with bacteria were the most predominant (80.6%). Gram-negative bacteria were the most relevant (1,748/2,650; 65.9%), followed by Gram-positive cocci (886/2,650; 33.4%). Members of Enterobacteriaceae, including *E.coli*, *K. pneumoniae*, *S. Typhi*, and the NTS were the most common Gram-negative causes of bacterial BSI in this hospital during 2010-2015. I report a high proportion of MRSA and ESBL-producing bacteria associated with both community and hospital acquired infections. I also highlight the circulation of MDR *Pseudomonas* and *Acinetobacter* in hospital acquired BSI in HTD. Therefore, antimicrobial treatment guidelines in HTD should be revised periodically to adapt to a new era with a high rate of antimicrobial resistance. *C. neoformans* and *T. marneffeii* (both fungal pathogens) were found in HIV-seropositive patients only. Therefore, fungal agents should be considered primarily when investigating BSI in this particular patient group.

2. I found that ESBL and AmpC lactamases were the two most common beta-lactamases produced by Gram-negative bacilli in BSI in this hospital. Common ESBL genes were *bla*_{CTX-M}, *bla*_{CIT}, and *bla*_{OXA}, while common AmpC genes were *bla*_{CIT}, *bla*_{DHA}, and *bla*_{EBC}. I also discovered a large proportion of organisms harbouring more than one ESBL gene, or ESBL and AmpC genes in

combination. Alarming, these resistance genes could be commonly found in both HAI and CAI BSI, which implicates the common circulation of resistant organisms in the community. These data emphasize the need for national surveillance for antimicrobial resistance and the implementation of antimicrobial stewardship in Vietnam immediately.

3. I found that NTS have replaced *S. Typhi* to be the most common *Salmonella* pathogens associated with BSI in HTD. The most common hosts for NTS were immunocompromised patients, particularly those infected with HIV or with cirrhosis. I conclude that iNTS disease is a severe infection in Vietnam, with a mortality rate (26%) comparable to that observed in parts of sub-Saharan Africa. I also highlight HIV infection as a major risk for both infection and death with iNTS in this setting. Although the sequence types of iNTS organisms identified in this study were common globally, I suggest continued surveillance to monitor for the presence of MDR sequence types, such as ST313, which has not, as of yet, been identified in Asia. I propose that HIV-infected hosts have a limited protective immune response to NTS. Using bacterial killing assays to compare the functional immune response in HIV infected and uninfected iNTS patients would be the next logical progression to these finding.
4. Lastly, by assessing the antimicrobial resistance profiles of 317 *Salmonella* via differing testing methods, I found that the *Salmonella* isolated here exhibited decreased susceptibility against the fluoroquinolones, which is the drug of choice to treat these infections. This observation suggests a requirement to evaluate the clinical effects of fluoroquinolones for treating *Salmonella* infections. I also report some problems in using the VITEK automatic

antimicrobial susceptibility system to screening susceptibility in *Salmonella*. E-test remains the best method for susceptibility testing, while the disc diffusion methods has a good performance for beta-lactams, azithromycin, and SXT.

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Appendix A: Protocol for Study 15EN

Project:

The bacterial etiology and antimicrobial susceptibility profile of bloodstream infections at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam

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Collaborating Institutions and Departments:

The Hospital for Tropical Diseases microbiology laboratory, HCMC, Vietnam

Oxford University Clinical Research Unit, HCMC Vietnam

Proposed Start Date:

February 2014

1. Introduction

This retrospective investigation is designed to study the aetiology, microbial population structure, antimicrobial susceptibility patterns, and antimicrobial susceptibility genes of

the bacteria causing bloodstream infections (bacteremia) at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City (HCMC), Vietnam. The main aims of this study are, i) to define the major causes of bacteremia in this setting, ii) investigate antimicrobial susceptibilities of the bacteria causing bloodstream infections and iii) investigate the pool of antimicrobial resistance genes in these organisms. We hypothesize that there is an emerging problem with antimicrobial resistance, particularly to third generation cephalosporins and carbapenems (Hoang et al., 2013; Thi Khanh Nhu et al., 2010; Trang et al., 2013), in this group of organisms. We hope that by studying and defining the antimicrobial susceptibility patterns we are able to develop an improved approach to managing bacteremia and we will use these data to initiate prospective studies of antimicrobial resistance in patients with suspected bacteremia.

This project will be a follow on from previously published studies of bloodstream infections at HTD (Baker et al., n.d.; Le et al., 2011; Nga et al., 2012; Wain et al., 1998).

2. Background

3. Aims and outcomes

3.1. Scientific rationale

There are limited contemporary data on the bacterial cause of bloodstream infections at HTD. We think that there is a dramatic surge in multi-drug resistant Gram-negative organisms and the emergence of Methicillin sensitive *Staphylococcus aureus* (MRSA). Therefore, to understand this important cause of morbidity and to best inform antimicrobial treatment regimes we aim to describe the bacterial etiology of bloodstream infection from 2010 to 2013 and investigate the circulation of antimicrobial

resistance genes through conventional molecular analysis, susceptibility testing, and genome sequencing.

3.2. Aims

This is a retrospective hospital study aiming to understand the cause of bacteremia and their associated antimicrobial susceptibility profiles at HTD. Our main interest lies in studying the Gram-negative organisms and MRSA and Methicillin sensitive *Staphylococcus aureus* (MSSA) causing bloodstream infections (Thwaites, 2010). However, to put these organisms into the context of bacteremia at HTD, and other locations, we will study all cause bacteremia retrospectively through patient records from HTD and the HTD microbiology laboratory. This study will be performed with a corresponding set of routine data and archived strains that is available over the last 4 years, i.e. from 2010 to 2013. We aim to investigate the major causes of bacteremia at HTD, their distribution by ward, their association with HIV, their antimicrobial susceptibility patterns, their content of antimicrobial resistance genes, their population structure, and their accessory genome structure.

3.2. Primary outcomes

1. A dataset describing etiology of bacteremia, with a corresponding strain collection, from 2010-2013 at HTD in HCMC.
2. An analysis of antimicrobial susceptibility profiles of the agents of bacteremia investigating study time trends and emerging resistance patterns.
3. A molecular analysis of the agents of bacteremia studying the distribution of the main antimicrobial resistance gene groups.

4. Genome sequences of pivotal strains from within this collection to study the population structure, the antimicrobial resistance gene pool, and other horizontally transferred DNA between bacteria causing bloodstream infections.

3.2. Secondary outcomes

1. An analysis of antimicrobial resistance profile and gene distribution by hospital ward to add insight into the circulation of bacteria causing nosocomial (hospital associated) infections.
2. An in depth screen of genes catalyzing resistance to the beta-lactams (specifically third generation cephalosporins and carbapenems) as these are the major agents to treat bacteremia at HTD.

3.3. Future outcomes after analyses

1. A comprehensive understanding of the microbiological and genetic makeup and the temporal and phylogenetic relationships of bacteria causing bloodstream infections at HTD.
2. A baseline of data permitting prospective studies aimed at detecting bacteremia cases more rapidly (i.e. by PCR on blood).
3. A baseline of data permitting prospective studies to detect patients early with multi-drug resistance infections of those with infections with bacteria containing ESBL/AMPC lactamase genes.
4. A data resource that will be made available to clinicians working at HTD managing bloodstream infections.

5. A measure of evolutionary rate, antimicrobial resistance development and the role of the accessory genome in the local evolution of the causes of bacteremia at HTD

4. Research plan

4.1. Study design

This is a retrospective, descriptive study conducted on data and strains collected over a four-year period from patients with bacteremia at HTD, activities will be broadly similar to those described previously (Nga et al., 2012).

4.2. Study sites

1. The Hospital for Tropical Diseases
2. Oxford University Clinical Research Unit

4.3. Required data and samples

1. Available routine microbiology laboratory data on bacteremia at HTD from 2010-2012 inclusive. These data will include:
 - i. Data on all cause bacteremia from 2010-2013 – e.g. etiology, date of isolation, ward, sex, HIV status and outcome
 - ii. Antimicrobial susceptibility patterns from all bacterial isolates
2. Access to all stored bacterial isolates that correspond to available data on bacteremia at HTD from 2010-2013. After re-culturing and identification these bacteria will be used for:
 - i. Additional antimicrobial susceptibility phenotyping

- ii. Molecular analysis for antimicrobial susceptibility genes
- iii. Genome sequencing of pivotal strains (defined by antimicrobial susceptibility data)
- iv. Additional bacterial phenotyping to investigate novel mechanisms of pathogenicity and anti microbial resistance identified by genome sequencing

5. Methods

5.1. Inclusion criteria

The analyzed data will be retrospective and the inclusion criteria for analysis will be every patient at HTD with a positive bacterial culture from a blood sample from the 1st January 2010 to 31st December 2013.

5.2. Exclusion criteria

There will be no exclusion criteria.

5.3. Study population

Systematic criteria concerning which patients should have blood cultures performed are not defined at HTD. However, in general, we consider that a blood culture is initiated by an admitting clinician in patients in whom a bloodstream infection is suspected on the basis of a fever ($>38^{\circ}\text{C}$) or the evidence of sepsis on the basis of the presence of two or more of the following features: fever ($>38^{\circ}\text{C}$) or sub-normal temperature ($<36^{\circ}\text{C}$); tachycardia (exact level according to age); tachypnea (exact level according to age); an elevated white cell count ($>12,000\text{ cells/mm}^3$) or depressed white cell count ($<4,000\text{ cells/mm}^3$) (Nga et al., 2012). We have no reason to believe there has been a systematic

change in the application of these criteria during the selected time course of the proposed study. Therefore, all patients with or without HIV admitted to the hospital who had a blood culture performed for suspected bloodstream infection from 1st January 2010 to 31st December 2013 will be included in this retrospective analysis.

Routinely, a member of the hospital staff records the date of blood draw, the patient's age, sex, and suspected diagnosis, the number of blood culture bottles inoculated, the result of the culture (whether positive or negative) and the susceptibility of the isolate to commonly used antimicrobial agents. Data from these records are subsequently entered into Excel (Microsoft office v12, Microsoft Corp, Redmond, WA, USA). These will be source data for this study. The number of patients admitted to the hospital annually will be obtained from hospital records.

5.4. Definition of bacteremia

A bloodstream infection episode in this study is defined as isolation of at least one clinically relevant pathogen from one blood culture, drawn from a patient with a clinical syndrome indicative of a bloodstream infection. Episodes will be identified from the electronic database as detailed above. As it is not practical to obtain and review the hospital records of all patients with a positive blood culture episode to ascertain whether a pathogen was clinically relevant for that particular patient, the following potential contaminant organisms will be excluded: coryneforms (*Corynebacterium*, etc.), coagulase-negative staphylococci, micrococci, *Propionibacterium*, *Bacillus*, alpha hemolytic streptococci, environmental Gram-negative bacilli, and non-pathogenic *Neisseria*. The overall contamination rate of these organisms will not be estimated in this study.

5.5. Study size

As this is retrospective descriptive study the sample size is dependent on the number of patients with available data according to the inclusion criteria during from 2010-2013

5.6. Sampling

All bacteria isolated during the outlined period of analysis will be studied for their antimicrobial susceptibility profiles and the presence/absence of antimicrobial resistance genes. Only selected organisms will be genome sequenced and will be selected prospectively on the basis of their susceptibility profiles and resistance gene content. Therefore, we cannot predict how many genomes will be sequenced.

5.7. Re-culturing and identification of bacteria

Typically, for blood culturing, HTD uses the BACTEC blood culture system, with 5–8 mL aliquots (for adults) and 2–5 mL aliquots (for infants and children) of venous blood inoculated into BACTEC plus aerobic bottles (Becton Dickinson). Inoculated BACTEC bottles are then incubated at 37 °C in a BACTEC 9050 automated analyzer for up to five days and sub-cultured when the machine indicates a positive signal. All sub-cultures are then performed onto fresh sheep blood agar, on chocolate agar if *Haemophilus influenzae* or *Neisseria meningitidis* is suspected, (media - Oxoid Unipath, Basingstoke, UK). Plates are incubated at 37 °C in air (for blood-agar), 5% CO₂ (for chocolate agar) for 48 hours. Organisms are then identified by standard microbiological methods including API20E identification kits (Bio-Mérieux, Craponne, France). All bacteria will

be re-cultured and checked as per the primary culture identification method. Any that are incorrect will be subjected to specific molecular testing (16s RNA) to ensure accuracy.

5.8. Antimicrobial susceptibility testing

When required for checking or to non-routine antimicrobials, antimicrobial susceptibility testing of the pathogens isolated will be performed by the disk diffusion method using guidelines established by the Clinical and Laboratory Standards Institute (CLSI) and, when required, by minimum inhibitory concentrations (MICs) by Etest or by VITEK automated machine. We will aim to generate a database of antimicrobial susceptibilities against Nalidixic acid, Ciprofloxacin, Ceftriaxone, Ceftazidime, Augmentin, Ampicillin, Trimethoprim-sulfamethoxazole, Azithromycin, Chloramphenicol, Imipenem and Amikacin for all Gram-negative organisms.

The production of extended-spectrum β -lactamases (ESBL) will be investigated using the double-disc synergy test by comparing zone sizes between ceftazidime discs against ceftazidime-clavulanic acid discs and cefotaxime discs against cefotaxime-clavulanic acid discs. Isolates with an increase in diameter of inhibitory zone of equal to or more than 5 mm by the synergy of clavulanate will be considered ESBL positive.

5.9. Isolation of nucleic acids

After re-culturing and identification, DNA will be extracted from all bacterial isolates using the Wizard Genomic DNA Extraction Kit (Promega, Fitchburg, USA). This is a standard method in the laboratories at OUCRU-VN. The quality and concentration of

the DNA will be assessed using a nano-drop spectrophotometer prior to PCR amplification and the Quant-IT Kit (Invitrogen, Carlsbad, CA) prior to DNA sequencing.

5.10. PCR for resistance genes

The primary focus study is to investigate the distribution of antimicrobial resistance genes in bacteria causing bloodstream infections at HTD. Therefore, all Gram-negative organisms will be subjected to PCR to detect genes catalyzing resistance to third generation cephalosporins, fluoroquinolones and carbapenems. Namely, all organisms will be subjected to conventional PCR for the following classes of resistance genes using previously described methods. The multiplex and monoplex PCRs are described in these publications (Dallenne et al., 2010; Parry et al., 2010; Pérez-pérez and Hanson, 2002; Thi et al., n.d.; Woodford et al., 2006), This panel of PCRs will be used; PCR1 - AmpC (MOX-1, MOX-2, CMY-1, CMY8-11), PCR2 - AmpC (LAT-1 to LAT-4, CMY2-7, BIL-1), PCR3 - AmpC (DHA1 and DHA-2), PCR4 - AmpC (ACC), PCR5 - AmpC (MIR-1T, ACT-1), PCR6 - AmpC (FOX-1-5b), PCR7 - ESB (CTX-M1), PCR8 - ESB (CTX-M2), PCR9 – ESB (CTX-M9), PCR10 - ESB (CTX_M8/M25), PCR11 - ESB (TEM), PCR12 – ESB (SHV), PCR13 – ESB (OXA1, 4, 30), PCR13 – qnrA, B, S, and PCR13 – gyrA, B, C.

5.11. Genome sequencing

Selected bacterial isolates will be sequenced at OUCRU-VN or at one of our collaborating genome sequences institutions (see later). Briefly, index-tagged paired end Illumina sequencing libraries will be prepared using one of 96 unique indexing tags as

previously described. These will be combined into pools of uniquely tagged libraries and sequenced on the Illumina Genome Analyzer GAII or HiSeq sequencer according to manufacturer's protocols to generate tagged 54-100 bp paired-end reads. This is a previously described for Gram-Negative organisms and *Staphylococcus* (Harris et al., 2013; Holden et al., 2013; Holt et al., 2013).

5.12. Data entry and storage

All available data will be entered onto an electronic database. Only the named investigators or their designee(s) will have access to this information. Other investigators from HTD and OUCRU-VN will be updated regularly and will be granted access to data when requested. Patients will not be identified by their names.

6. Analysis plan

6.1. Statistical comparisons

Data will be presented in the form of tables and bar charts for descriptive variables i.e. number of specific organisms per year and number of resistant organisms per year. Time trends (over the 4-year period), including the proportion of cultured isolates by year, the antimicrobial susceptibility patterns and HIV positivity rate will be determined by logistic regression, and odds ratios will be presented in units of time (per year). All statistical analysis will be performed using Stata version 11 (StataCorp LP, College Station, TX, USA); and p-values of ≤ 0.05 will be considered significant.

6.2. Antimicrobial resistance genes

The presence/absence of antimicrobial resistance genes will be reported as proportions per organism and then stratified by organism, year, and hospital ward.

6.3. Genome sequencing

Genome sequences will be analyzed to study phylogenetic relationships, the presence/absence of genes and also antimicrobial resistance gene content and firstly analyzed by species and then a group of Gram-negative organisms. Briefly, for phylogenetic analysis, chromosomal Single Nucleotide Polymorphism (SNP) alleles will be concatenated for each strain to generate a multiple alignment of all SNPs. For maximum likelihood (ML) analysis, RAxML will be run 10 times using the generalized time-reversible model and one thousand bootstrap pseudo-replicate analyses were performed to assess support for the ML phylogeny. Root-to-tip branches will be extracted from the ML tree using the program TreeStat. The relationship between root-to-tip distances and year of isolation were analyzed using linear regression. For BEAST analysis (v1.6), a GTR+ Γ substitution model and defined tip dates, as the date of isolation will be used (Harris et al., 2013; Holden et al., 2013; Holt et al., 2013).

To detect the presence or absence of genes read sets will be assembled using the de novo short read assembler Velvet and Velvet Optimizer. Strain specific read sets will then be aligned to the pan-genome. Taxonomic investigation of accessory and resistance genes will be performed using MG-RAST v3.2 (<http://metagenomics.anl.gov>).

7. Sample shipments

7.1. Sample shipment and overseas investigations

Investigations in part of this study may take part at HTD, OUCRU-VN or other collaborating institutions internationally i.e. samples will be required to be sent overseas for laboratory analysis outside current laboratory capabilities of HTD and OUCRU-VN to the collaborating institutions. All investigators will be informed on any material leaving HTD/OUCRU-VN and appropriate requests to regulatory authorities will be made. The current locations identified for collaboration include; the Wellcome Trust Sanger Institute, the University of Sydney Australia and the University of Melbourne Australia. The material sent overseas for this study will constitute in the primary incidence nucleic acid for DNA sequencing only as HTD/OUCRU-VN currently does not have the capacity for large-scale bacterial genome sequencing. This material will be sent under MOU's and collaboration agreement between OUCRU-VN and these institutions. HTD/OUCRU-VN will remain the owners of all sequence data and will oversee all analyses. Therefore collaborations to achieve the aims of this protocol are essential Depending on the genomic sequences, bacteria may also need to be shipped for secondary analysis or phenotyping, as HTD/OUCRU-VN currently does not have the capacity for high-throughput phenotyping. For other studies outside this protocol, additional IRB approval will be sought.

7.2. Future investigations at HTD/OUCRU-VN

Additional investigations may be performed on isolated nucleic acids and bacteria collected as part of this study. These samples will only be used to study the aetiology, the epidemiology, or the phenotype of these organisms and will occur in the HTD microbiology laboratory or the OUCRU-VN laboratory of Dr Stephen Baker and will not be distributed to other investigators in Vietnam without permission from all

collaborating institutions. The principal investigators will make decisions on these studies. For other studies outside this protocol, additional IRB approval will be sought.

8. Protection of human subjects

8.1. Use of stored human specimens

No human samples will be used in this study; this study will only investigate the bacteria cultured from those with bacterial bloodstream infection.

8.2. Long term storage of data

Data will be protected in the custody of HTD/OUCRU-VN under joint ownership of HTD and OUCRU-VN. Data will be stored in password-protected computer servers, which will be located in locked rooms. Only investigators or their designee(s) will have access to the data, all data will be identified by an individual patient identification code and will be anonymous.

8.3. Long term storage of specimens

The HTD microbiology laboratory currently holds and stores the samples for this study and this collection under an HTD SOP HTD. Nucleic acid extractions will be stored until all analyses for this study have been performed and then destroyed when all molecular studies are complete.

8.4. Study withdrawal

This is a retrospective study and patients will not be prospectively enrolled.

8.5. Risks and benefits

This is a no patient risk study because it does not involve any investigational new drugs or interventions. The collection of all biological samples for use in this study have been performed as part of a clinical assessment and are consistent with the local standard of care and good clinical practice.

8.6. Regulation and quality assurance

The study will be conducted in compliance with this protocol, relevant sections of the International Conference on Harmonization Good Clinical Practice (ICH GCP) guidelines and any applicable regulatory requirement(s). Quality assurance and quality control procedures will be implemented for all data collection, documentation, and specimen handling.

8.7. Institutional review board/ethics committee

This protocol and the relevant supporting information will be submitted to the EC/IRB of HTD for review and will not be initiated at that site until after approval. Any amendments will also need to be approved by HTD IRB/IEC prior to implementing changes in the study.

8.8. Informed consent process

This is a retrospective analysis of bacteria and data collected as part of a routine microbiological provision for diagnosis, there is no requirement for informed consent.

8.9. Participant confidentiality

1. All data will be stored securely at the study site in locked file cabinets or password protected devices in areas with access limited to study staff.
2. All specimens, reports, study data collection, process, and administrative forms will be identified by a coded number.
3. Study databases will be secured with password-protected access systems and controlled distribution web-based security certificates.
4. No identifying information will be included in publications or presentations resulting from this work.

8.10. Data handling and record keeping

1. The investigator is responsible for maintaining all study records. The investigator is responsible for the timeliness, completeness and accuracy of the information in the original dataset and the clinical data management system.
2. HTD/OUCRU-VN staff will enter data into computers, which will upload data securely to an Internet-based database.
3. Laboratory staff will record specimens (and their aliquots), their storage location, their shipments using a central commercial database system (Freezerworks).

4. All necessary tools, instruction, and training will be provided to all site staff involved in data entry to ensure the correct and consistent completion database prior to the study starting.

8.11. Study records retention

Data from this study will be entered into an electronic database in password-protected computer servers, which will be located in locked rooms. Only investigators or their designee(s) will have access to the data, all data will be identified by an individual patient identification code and will be anonymous. These data will be stored indefinitely.

9. Publication plan

Data from this study is of substantial interest to the scientific and clinical research communities. Therefore, we aim to publish a number of manuscripts from this work in international peer-reviewed journals. Additionally, these data will contribute to the data required for Dr Nguyen Phu Huong Lan's PhD thesis at the Open University UK and a number of HTD/OUCRU-VN collaborative MSc projects. As this is a HTD/OUCRU-VN collaboration then HTD and OUCRU authors will both be recognized in predominant authorships, these will be principal investigators. Manuscripts that may be published from this work will be comprised of the following data.

1. General description of the bacterial causes of bloodstream infections and their antimicrobials susceptibility profiles at HTD.
2. The presence absence of the outlined antimicrobial resistance genes.
3. The emergence of methicillin resistant *Staphylococcus aureus* in Vietnam.

4. The accessory gene pool of Gram–negative organisms causing bloodstream infections at HTD

10. Support

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Appendix B: Protocol for Study 09EN

Project:

Invasive nontyphoidal *Salmonella* in Hospital for Tropical Diseases

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1. Introduction

This is a retrospective study to describe epidemiological, clinical features and bacterial characteristics of bacteremia cases due to nontyphoidal *Salmonella* in Hospital for Tropical Diseases from 2008 to 6/2013.

2. Background

Salmonella are gram- negative bacilli that belong to the family *Enterobacteriaceae*. The genus *Salmonella* was named after Daniel Elmer Salmon, who firstly described this type of organism as a pathogen in 1884.

It is reported that there were 22 milion cases of typhoid fever annually around the world with 200.000 mortality cases (Bhutta and Threlfall, 2009). According to a global analysis in 2008, the highest incidence of typhoid (>100 cases per 100.000 population annually) is in South Central Asia, Southeast Asia and Southern Africa (Bhutta and Threlfall, 2009). The crude incidence of typhoid cases was as high as in Southern Africa (233 cases/ 100.000 population annually) and Southern central Asia (622 cases/ 100.000 population annually).

The medium high incidence (10-100 cases/ 100.000 population annually) is in the rest of Asia, Africa, Latin America, Caribbean islands and Oceania. Whereas the lowest incidence (<10 cases per 100.000 population per year) was documented in Europe, North America, Australia and New Zealand in which most cases were associated with travelling to epidemic areas.

Although Vietnam is located in Southeast Asia, the medium incidence of typhoid from 1991 to 2011 is around 23.2/100.000 per year which is not as high as some epidemic areas in the region. A data from Hochiminh city-Vietnam shows that *Salmonella* Typhi is the dominant pathogen in blood stream infection until 2002 (Nga et al., 2012). Since then, the number of infection has been declined steadily about 30% per year.

In contrast, NTS diseases is not as common as typhoid. There is no global epidemic of invasive NTS infection until now. Therefore, studies of NTS are still limited. As far as we know, infection of *nontyphoidal Salmonella* (NTS) has two main types: gastroenteritis and invasive diseases(de Jong et al., 2012). People get sick after they consumed a large amount of nontyphoidal *Salmonella* bacteria. The clinical manifestation can be a self-limited gastroenteritis disease or develop to a septicaemia (the most frequent type of NTS invasive diseases) in 10% cases. Blood-stream infection of NTS species is found in those risk groups such as immunocompromised individuals, HIV patients, malnutrition children(Feasey et al., 2012; Melita A Gordon et al., 2002; Khan et al., 2010)... It is reported that 93.8 million cases of NTS infection every year in the world with 155.000 fatal cases (13).

Enteric infections caused by NTS have similar clinical manifestations to those induced by other enteric bacteria, and thus can rarely be distinguished based on presenting clinical characteristics. NTS-induced gastroenteritis is characterised by abdominal pain, fever, watery diarrhoea, and occasionally mucoid or bloody diarrhoea. Vomiting and/or nausea occur frequently but are not severe or protracted. The incubation period is varied, depending on the host and bacterial serotype, but typically ranges from 6 to 72 hours. The disease is usually self-limiting but can be more severe in the very young and elderly or immunocompromised, with a typical duration of illness of 4 to 7 days.

The international study in ASIA revealed that nine serogroups (B, C1, C2, D, E, G, I, K, and M) were responsible for NTS gastroenteritis disease(Majowicz et al., 2010). It is also found that serogroup B and D were the two commonest in most of the research

sites. Serotype *S. enterica* serotype Choleraesuis and *S. Virchow* were the predominant serotypes based on the study result.

NTS gastroenteritis imposes a substantial burden in both developed and developing regions (Majowicz et al., 2010; Voetsch et al., 2004). *Salmonella*-associated gastroenteritis is estimated to be responsible for a globally annual number of 93.8 million cases and 155 thousands deaths; 86 % of all cases are thought to be foodborne infections (Majowicz et al., 2010). In the US, FoodNet surveillance has estimated that nearly 1.4 million persons are infected with NTS annually, resulting in 168,000 clinic visits, 15,000 hospitalisations and 400 deaths (incidence: 12.3 cases per 100,000 population) (Voetsch et al., 2004). Whereas in the UK, there were 41,000 infection of NTS each year (population 60 million) resulting in 1,500 hospitalization and 119 deaths (Zaidi et al., 2006). A case series in Spain showed a mortality rate of 12.2% in adults which most of them were suffered from septic metastatic diseases (Gordon, 2011). Outbreaks of NTS gastroenteritis are common and often of zoonotic origin (Voetsch et al., 2004). Severe NTS infections are more frequently observed in resource-limited countries, with mortality rates ranging from 18 to 24 %.

In these severe illnesses, antimicrobials should be prescribed. There is no effective vaccine against NTS; hence, disease prevention and control programs mostly rely on improving personal hygiene and sanitation with particular attention paid to food safety. About 5 % of all cases of NTS gastroenteritis may progress into bacteraemia and require medical attention. Even more dramatic are NTS bacteraemia that develop without any apparent gastrointestinal disease.

The epidemiology of invasive NTS infections is highly geographical and common in parts of sub-Saharan Africa. Over the past 10-15 years this disease has come to forefront of *Salmonella* research, lead mainly by groups in Kenya, Malawi and Tanzania

(Gordon, 2011). It appears that invasive NTS disease is causing a regional epidemic in sub-Saharan Africa and is associated strongly with HIV and also Malaria. The disease appears to affect children and can have a mortality rate as high as 40% in some locations(Gordon, 2011) .Additionally, a systemic review on blood culture positive patients in Africa showed that NTS organisms accounted for 17% of 5,578 positive samples and associated with HIV infection while *S. Typhi* were found at a lower rate (9.9%) and mostly in non HIV infected individuals. *S. Typhimurium* and *S. Enteritidis* are the two common serovars causing NTS infections in these locations(Gordon, 2011)

Due to the fact that very few study of invasive NTS infection have been performed in Asia, information of clinical symptoms of this disease is mostly described in the Sub-Saharan African population. The common illness caused by NTS serovars in this population is similar to general symptoms of bacteraemia s. Namely, a high fever is frequently observed while diarrhoea and other gastrointestinal complaints are rare. Pneumonia is also usually associated with NTS but can be caused by co-infections organisms such as *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* (Gordon, 2011). Splenomegaly is also frequently a clinical sign associated with invasive NTS infection, but this is not specific. Meningitis may also be associated with infection in the sub-Saharan African population but this is not as common as sepsis, but NTS strains have been reported as a second most common cause of bacterial meningitis in Malawi. NTS meningitis is associated with schistosomiasis, it is hypothesis that the parasite carries the bacteria to the central nervous system. Notably, and unlike enteric fever the fatality rate of NTS meningitis and NTS bacteraemia is very high in both children and adults.

Available data of invasive NTS in Asia remains limited both in number and with respect to the disease in different patient groups. A multi-centre study in Asian people conducted under 15 years old showed that NTS accounted for less than 1% (only 6 cases/ 20,537 total blood samples) of all the febrile pathogens (Khan et al., 2010). Children accounted for five of these six NTS infections, there were only one adult infected. Karachi (Pakistan) had the highest incidence of (7.2/100,000 population) of invasive NTS while Hue (Vietnamese study site) had no invasive NTS case. This study did collect data regarding the co-infection with HIV or malaria but the incidence of these diseases are considerably lower than in an equivalent age distributed population in sub-Saharan Africa. In the six invasive NTS cases, group D *Salmonella* were isolated in all of the three cases from Pakistan, one isolate from each *Salmonella* group A, C, E were from India and Indonesia.

Invasive non-typhoidal *Salmonella* infections in Vietnam

The only existing data regarding invasive NTS infections in Vietnam arises from a 15 years- retrospective study of blood culture data performed at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam (Nga et al., 2012). In this study, amongst 66,111 blood cultures performed, a clinically relevant pathogen was isolated in 7,645 episodes (positivity rate; 116/1,000 cultures). *S. Typhi* was the predominant pathogen until 2002; however, a considerable annual decline in the proportion of *S. Typhi* was observed over the period of investigation (OR 0.6993, 95% CI [0.6885, 0.7103], $p < 0.0001$). Conversely, there was a significant increase in the proportions NTS organisms, *Cryptococcus neoformans* and *Penicillium marneffei*, concurrent with increasing HIV prevalence. This paper documented a substantial longitudinal shift in bloodstream infection etiology in southern Vietnam. We proposed that such changes were related to

increasing economic prosperity and HIV prevalence, and this pattern marks a substantial change in the epidemiology of invasive salmonellosis in Southeast Asia.

3. Aims

To describe epidemiological characteristics, clinical features of all NTS bacteremia cases from 2008 to 6/2013

To describe the different clinical features, treatment and outcome between HIV and non- HIV patients.

To describe the antibiotic susceptibility patterns of nontyphoid *Salmonella*, how it correlates to treatment and outcome, and in comparison with *Salmonella* Typhoid at the same period of time.

To describe the phenotype distribution of nontyphoid *Salmonella*.

4. Research plan

4.1. Study design

The study is performed in Hospital for Tropical Diseases (HTD), the reference hospital for infectious diseases in Hochiminh city and the South of Vietnam. It is a 550 beds-setting that receives adult and children, including HIV patients.

This is a retrospective and descriptive study to all NTS bacteremic patients in HTD including patient charts and NTS strains from 2008 to 6/2013.

4.2. Study sites

1. The Hospital for Tropical Diseases
2. Oxford University Clinical Research Unit

4.3 Method:

Protocol of this study was submitted to the Ethical and Scientific Committee of HTD in 2012 and got approval in 2012. By that I have the permission to access to all study patient charts that kept in the Hospital storage. I developed a special clinical case report for inputting all necessary information from the patient charts. I am also permitted to access to all NTS data kept in the logbooks of Microbiology laboratory, to use the all the NTS strains stored in freezers of Microbiology laboratory for identification, antibiotic susceptibility testing and molecular analysis.

4.4 Data and Samples

- Patient data which obtained from the patient charts, and would be recorded in patient record form.
- Information of NTS strains and *Salmonella* Typhi strains that kept in HTD Microbiology laboratory.
- NTS strains from the storage of HTD Microbiology laboratory:
 - Reculturing and reidentify, re –examine the antibiotic susceptibility of NTS strains in the study period.
 - Molecular analysis: MLST to phenotype all NTS strains, detect resistant genes if available.

4.5 Inclusion and Exclusion criteria

-This is a retrospective study and the inclusion data is for all the patients that have blood culture positive with nontyphoid *Salmonella* strain from 2008 to 6/2013

-There is no exclusion criteria.

4.6 Sample size

Because NTS infection is not a popular infection in Southeast Asia and HTD, the number of more than 100 for patient charts and survival NTS strains is expected to be enough for analysis.

4.7 Reculturing and identification of Nontyphoidal *Salmonella*

All nontyphoidal *Salmonella* as well as all the pathogens isolated from the blood culture were kept routinely in BHI glycerol at -20 C in Microbiology storage stock. We have recultured all the stored isolates into MacConkey agar. We used the second subcultured generation for identification and antimicrobial susceptibility testing.

-Identification of *Salmonella* is based on routine biochemical testing and antisera test.

4.8 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing will perform for all *Salmonella* nontyphoid isolates. Testing media were Muller-Hinton agar and antibiotic paper disks were from Oxoid, USA. Performance procedure, quality of media and antibiotic disks were all checked with all the standard quality control of HTD Microbiology laboratory that were accredited with ISO 15189-2008. Antibiotic testing includes: ampicillin, ceftriaxone,

cefepim, ofloxacin, ciprofloxacin, trimethoprim-sulfamethoxazole, chloramphenicol, amikacin and imipenem and other antimicrobials.

4.9 MLST

MLST will be performed to phenotype all NTS strains.

4.10 Data entry and storage

All available data will be entered onto an electronic database. Only the named investigators or their designee(s) will have access to this information. Patients will not be identified by their names.

4.11 Data analysis

Data will be presented in the form of tables and bar charts for descriptive variables. All statistical analysis will be performed using Stata version 11 (StataCorp LP, College Station, TX, USA); and p-values of ≤ 0.05 will be considered significant.

5. Protection of human subjects

5.1. Use of stored human specimens

No human samples will be used in this study; this study will only investigate the bacteria cultured from those with bacterial bloodstream infection.

5.2. Long term storage of data

Data will be protected in the custody of HTD/OUCRU-VN under joint ownership of HTD and OUCRU-VN. Data will be stored in password-protected computer servers, which will be located in locked rooms. Only investigators or their designee(s) will have access to the data, all data will be identified by an individual patient identification code and will be anonymous.

5.3. Long term storage of specimens

The HTD microbiology laboratory currently holds and stores the samples for this study and this collection under an HTD SOP HTD. Nucleic acid extractions will be stored until all analyses for this study have been performed and then destroyed when all molecular studies are complete.

5.4. Study withdrawal

This is a retrospective study and patients will not be prospectively enrolled.

5.5. Risks and benefits

This is a no patient risk study because it does not involve any investigational new drugs or interventions. The collection of all biological samples for use in this study have been performed as part of a clinical assessment and are consistent with the local standard of care and good clinical practice.

5.6. Regulation and quality assurance

The study will be conducted in compliance with this protocol, relevant sections of the International Conference on Harmonization Good Clinical Practice (ICH GCP) guidelines and any applicable regulatory requirement(s). Quality assurance and quality control procedures will be implemented for all data collection, documentation, and specimen handling.

5.7. Institutional review board/ethics committee

This protocol and the relevant supporting information will be submitted to the EC/IRB of HTD for review and will not be initiated at that site until after approval. Any amendments will also need to be approved by HTD IRB/IEC prior to implementing changes in the study.

5.8. Informed consent process

This is a retrospective analysis of bacteria and data collected as part of a routine microbiological provision for diagnosis, there is no requirement for informed consent.

5.9. Participant confidentiality

1. All data will be stored securely at the study site in locked file cabinets or password protected devices in areas with access limited to study staff.
2. All specimens, reports, study data collection, process, and administrative forms will be identified by a coded number.
3. Study databases will be secured with password-protected access systems and controlled distribution web-based security certificates.

4. No identifying information will be included in publications or presentations resulting from this work.

5.10. Data handling and record keeping

1. The investigator is responsible for maintaining all study records. The investigator is responsible for the timeliness, completeness and accuracy of the information in the original dataset and the clinical data management system.

2. HTD/OUCRU-VN staff will enter data into computers, which will upload data securely to an Internet-based database.

3. Laboratory staff will record specimens (and their aliquots), their storage location, their shipments using a central commercial database system (Freezerworks).

4. All necessary tools, instruction, and training will be provided to all site staff involved in data entry to ensure the correct and consistent completion database prior to the study starting.

5.11. Study records retention

Data from this study will be entered into an electronic database in password-protected computer servers, which will be located in locked rooms. Only investigators or their designee(s) will have access to the data, all data will be identified by an individual patient identification code and will be anonymous. These data will be stored indefinitely.

6. Publication plan

Data from this study is of substantial interest to the scientific and clinical research communities. Therefore, we aim to publish a number of manuscripts from this work in international peer-reviewed journals. Additionally, these data will contribute to the data required for Dr Nguyen Phu Huong Lan's PhD thesis at the Open University UK and a number of HTD/OUCRU-VN collaborative MSc projects. As this is a HTD/OUCRU-VN collaboration then HTD and OUCRU authors will both be recognized in predominant authorships, these will be principal investigators.

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Case report form

Clinical characteristic and epidemiology of Nontyphoidal *Salmonella* infection in Hospital for Tropical Diseases from 2008 to 2013

DEMO	
Study number: [][][][]	Patient Initials [][][][][]

I. Demographic data:

1. Date of birth: [][][]/[][][]/[][][] (dd/mm/yy)
2. Hospital number: []/[][][]
3. Gender: ☐ Male ☐ Female
4. Ethnicity: ☐ Kinh ☐ Other : _____
5. Occupation: ☐ Farmer ☐ Seller ☐ Driver
☐ Worker ☐ Officer
☐ Unemployed ☐ Other: _____
6. Address: []
7. District: []
8. Province/City: []
9. Date of admission: [][][]/[][][]/[][][] (dd/mm/yy)
10. Ward of admission: ☐ ICU ☐ Ward E ☐ Ward A ☐ Other.....
11. Where did the patient live: []
12. HIV status: ☐ Yes ☐ No
If yes, day of diagnosis: [][][]/[][][]/[][][] (dd/mm/yy)
13. Hepatitis: ☐ Yes ☐ No
☐ Hepatitis B ☐ Hepatitis C ☐ Alcoholic cirrhosis ☐ Other :
14. Other underlying disease : ☐ Yes ☐ No
15. If yes : ☐ Diabetes ☐ Cancer ☐ Steroid medication/suppression medication
☐ Autoimmune disease
☐ Other : (please specify)

MEDHIST	
Study number: [][][][][][][][]	Patient Initials [][][][][][]

1. Day of illness : [][]

2. Reason for admission :

- | | |
|-----------------------------|--------------------------------|
| a. Fatigue | <input type="checkbox"/> |
| b. Fever | <input type="checkbox"/> |
| c. Cough | <input type="checkbox"/> |
| d. Anemia | <input type="checkbox"/> |
| e. Cachexia | <input type="checkbox"/> |
| f. Shock | <input type="checkbox"/> |
| g. Jaundice | <input type="checkbox"/> |
| h. Headache | <input type="checkbox"/> |
| i. Abdominal enlargement... | <input type="checkbox"/> |
| j. Diarrhea | <input type="checkbox"/> |
| k. Abdominal pain | <input type="checkbox"/> |
| l. Chest pain | <input type="checkbox"/> |
| m. Dyspnoea | <input type="checkbox"/> |
| n. Dizziness | <input type="checkbox"/> |
| o. Confusion | <input type="checkbox"/> |
| p. Nausea | <input type="checkbox"/> |
| q. Anorexia | <input type="checkbox"/> |
| r. Weightloss | <input type="checkbox"/> |
| s. Cold/Shivery | <input type="checkbox"/> |
| t. Other: | <input type="checkbox"/> _____ |

3. Treatment prior admission : ☐ HIV clinic
☐ Liver clinic
☐ Private clinic
☐ Other hospital
☐ Don't know
4. Receiving ARV: ☐ Yes ☐ No ☐ Don't know If yes, time: [__][__] day
5. Cotrimoxazole medication: ☐ Yes ☐ No ☐ Don't know
If yes, time: [__][__] month
6. Antibiotic treatment prior admission: ☐ Yes ☐ No
7. Risk factors :
- a. Injection drug user..... ☐ Yes ☐ No
8. a. CD4: [__][__][__] b. % CD4: [__][__] c. CD8: [__][__][__]
9. Tuberculosis : ☐ Yes ☐ No Time: [__][__]/[__][__]/[__][__]
10. P.marneffeii coinfection: ☐ Yes ☐ No Time: [__][__]/[__][__]/[__][__]
11. C.neoformans coinfection.... ☐ Yes ☐ No Time: [__][__]/[__][__]/[__][__]
12. Dermatomycosis:..... ☐ Yes ☐ No Time: [__][__]/[__][__]/[__][__]
13. Other:[_____] Time: [__][__]/[__][__]/[__][__]

EXAM	
Study number: [][][][][][][][]	Patient Initials [][][][][]

1. Blood pressure:[][][]/[][], Pulse: [][][], Respiratory rate: [][],
Weight: [][] kg

2. Fever: ☐ Yes ☐ No Highest temperature: [][]
 - a. If yes, please specify: ☐ Continuous fever
 - ☐ Intermittent fever
 - ☐ Mild fever
 - ☐ Sometime

3. Habitus: ☐ Normal ☐ Cachexia ☐ Emaciated ☐ Oedema
4. Anemia: ☐ Yes ☐ No
 - a. If yes, ☐ Severe ☐ Moderate ☐ Mild
5. Skin appearance:..... ☐ Normal ☐ Jaundice ☐ Paleness
6. Lymph nodes: ☐ Armpit ☐ Neck ☐ Poop ☐ Other_____
7. Respiratory rate: ☐ Normal breath rate ☐ Abnormal

If abnormal: ☐ Tachypnea

☐ Dyspnea

☐ Respiratory failure

☐ Rale

☐ Pleural infusion

☐ Pneumothorax

☐ Other_____
8. Cardiovascular:..... ☐ Normal ☐ Abnormal

If abnormal, please specify: [_____]

[_____]

9. Digestive system: ... ☐ Normal ☐ Abnormal

If abnormal, please specify:

- ☐ Hepatomegaly
- ☐ Splenomegaly
- ☐ Ascites
- ☐ Abdominal lymphadenopathy
- ☐ Other _____

10. Central nervous system: ☐ Normal ☐ Abnormal

- ☐ Meningismus
- ☐ Focal neurologic signs _____
- ☐ Other _____

11. Ear throat nose :..... ☐ Normal ☐ Abnormal

If abnormal, please specify: [_____]
[_____]

12. Other signs:

[_____]
[_____]
[_____]

LAB	
Study number: [][][][][][][][]	Patient Initials [][][][][]

1. Hct (Hb):
 - a. At admission:[][][]% ([][][] g/dL)
 - b. At discharge:.....[][][]% ([][][] g/dL)

2. Leukocyte:[][][] K/uL
 (N[][][]% M[][][]% E[][][]% L[][][]%)

3. Platelets :[][][] K/uL
4. Creatinin:[][][] umol/L (Male: 62-120, Female: 53-100 umole/L)
5. AST:[][][] (< 37 U/L).
6. ALT:[][][] (< 40 U/L)
7. GGT:[][][] (Male: 11-50 U/L, Female: 7-32 U/L)
8. Electrolyte test:
 - a. Na:[][][] mmol/L (135-145 mmol/L)
 - b. K:[][] mmol/L (3.5-5 mmol/L)
 - c. Ca:[][] mmol/L (2.15-2.6 mmol/L)

9. Xray chest: ☐ Normal

☐ Tuberculosis
☐ Pneumocystic
☐ Pneumonia
☐ Pleural infusion
☐ Pneumothorax
☐ Other _____

10. Ultrasound: ☐ Hepatomegaly Size:

☐ Splenomegaly
☐ Ascites
☐ Abdominal lymphadenopathy
☐ Other _____

11. a. CD4: [][][][] b. %CD4: [][][][] c. CD8: [][][][]

12. Stool direct examination: ☐ Yes ☐ No
- ☐ Erythrocyte
- ☐ Leukocyte
- ☐ Enteric parasites.....
13. a. Stool culture:..... ☐ Positive ☐ Negative
- If positive:..... ☐ *Salmonella spp* ☐ Other_____
- b. Cultured day: []/[]/[] c. Grown day: []/[]/[]
- d. Identification day: []/[]/[]
14. a. Blood culture:
- ☐ Positive
- ☐ Coinfection_____
- b. Culture day: []/[]/[] c. Grown day: []/[]/[]
- d. Identification day: []/[]/[]
- e. Serotype: Serology:.....
- MLST type:.....

15. Antibiotic resistant pattern:

	AMP	AUG	AZI	CRO	C	CIP	CN	NA	OFL	SXT	CAZ	ESBL
Resistant												
Intermediate												
Sensitive												

16. Other laboratory testing:

[_____]

[_____]

PROGRESSION	
Study number: [][][][][][][][]	Patient Initials [][][][][][][]

6. Progression: ☐ Discharge ☐ Improvement ☐ Worsening
☐ Death ☐ Nonassessable

7. Improvement or discharge:

- a. Fever clearance day: [][]/[][]/[][]
b. Day of recovery response: [][]/[][]/[][]
8. Day of discharge:..... [][]/[][]/[][]
9. Duration of stay:[]
10. Other note :